BIOCHEMISTRY OF
AUTOTROPHIC BACTERIA
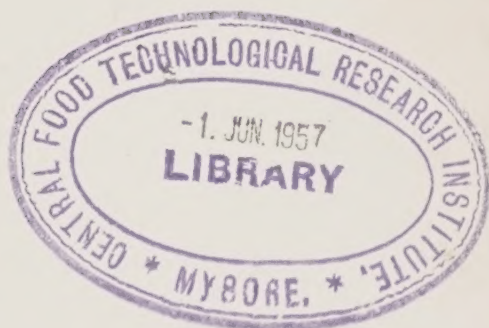
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LEES

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By

HOWARD LEES, Ph.D.

Department of Biological Chemistry,
University of Aberdeen

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CONTENTS

To Dad

PREFACE

Autotrophic bacteria are not adequately dealt with in any textbook of microbiological biochemistry; as a consequence the interesting biochemical problems they present tend to be skated over or ignored by university students of biochemistry and (to be frank) by their lecturers as well. This book is an attempt to remedy that state of affairs. Primarily it is meant to present the autotrophic bacteria in a comprehensible way to university students taking an ordinary or honours degree in biochemistry ; because it is meant for these students, the accent of the book is entirely biochemical, the morphology and ' general bacteriology ' of the organisms are no more than touched on.

If it be objected that our present knowledge of the biochemistry of the autotrophic bacteria is too fragmentary to make a book, I should reply, with Boswell, ' If a man is to wait till he weaves anecdotes into a system, we may be long in getting them, and get but few in comparison of what we might get '*. Indeed, in Chapter 8 I have attempted to ' weave the anecdotes into a system ' ; but whether the system is an acceptable one only future research will decide.

I must acknowledge my debt to Professor J. H. Quastel, F.R.S., who first stimulated and nurtured my interest in autotrophic bacteria. I also owe much to discussions with Professor T. Y. Kingma Boltjes, Dr. Jane Meiklejohn, Dr. H. G. Thornton, F.R.S., Professor M. D. Appleman, Dr. H. L. Jensen, and Mr. J. R. Simpson. The last three are also to be thanked for carrying out the tedious task of reading the manuscript and indicating errors; any that remain are my own.

Above all I must pay tribute to the gentle and kindly advice, help and encouragement of Professor W. O. Kermack, F.R.S. Few who have worked with him can have failed to profit from

* *Journal of a Tour to the Hebrides*, Entry for 15 August 1773.

vii

his scientific insight and precision ; none can have remained impervious to his happiness and charm. All will have from time to time forgotten (as those of us who still work with him continue to forget) how many of ' our own ' scientific ideas were originally his.

HOWARD LEES

Aberdeen,
February 1955

1

INTRODUCTION

THE existence of autotrophic bacteria is easily demonstrated. Dissolve two or three grammes of acid potassium phosphate in a litre of tap water, add one drop of pharmaceutical (B.P.) 'dialysed iron', and adjust the pH to about 7·5 with caustic soda ; add two or three grammes of calcium carbonate and half a gramme of sodium nitrite. Place 50 c.c. of this preparation in each of two quarter-litre conical flasks. To the contents of one flask add a knife-point of garden soil. Plug the mouths of both flasks with cotton wool (to prevent the entry of dust and to reduce evaporation) and place the flasks in a dark cupboard, at room temperature. If the contents of the flasks are analysed after about a month, it will be found that in the flask without soil the nitrite concentration is virtually unchanged. In the flask to which soil was added there will be no nitrite, instead there will be a roughly equal quantity of nitrate. Moreover, it will be found that one or two c.c. of the contents of this flask, added to a fresh 50 c.c. of the original potassium phosphate + calcium carbonate preparation, will also induce therein, after a suitable lapse of time, the same conversion of nitrite to nitrate. This cascade may be continued indefinitely. At any stage, a microscopical examination of the contents of a flask will show the presence of small bacteria congregated round the calcium carbonate particles. The medium is entirely inorganic ; after a few transfers, the carry-over of possible food materials from the original knife-point of soil is virtually nil. Here are bacteria living, growing, multiplying, in a medium apparently devoid of foodstuffs. The question arises : What are they living on ?

The answer is that after a few transfers there is a rather crude culture of the autotrophic bacterium *Nitrobacter* and its 'food', in one sense, is nitrite which it oxidizes (by means of atmospheric oxygen) to nitrate. This reaction releases energy which

Nitrobacter taps and uses to effect the reduction of atmospheric carbon dioxide so that the carbon of the carbon dioxide becomes the carbon of the organic material of the cell constituents. The ' foods ' of *Nitrobacter*, apart from a few mineral elements, are simply nitrite, oxygen, and carbon dioxide. It is an ' autotrophic ' bacterium, that is a bacterium which may be defined in a preliminary way as one that can live, grow, and multiply, in an environment totally free from organic compounds. There are many such bacteria ; some use the energy released by an inorganic oxidation (as *Nitrobacter* does), some use light energy, all reduce carbon dioxide to a level from which it can be transformed into the bewildering variety of organic compounds that go to make up a living cell, none requires any ' food ' other than mineral elements, a source of energy (light or some inorganic compound) and a supply of carbon dioxide.

There are several ways of studying the activities of these bacteria. They may be isolated in bacteriologically pure culture and grown either in purely mineral liquid media, or on mineral media solidified either by silica gel or by agar. They may be grown in enrichment cultures produced by repeated subculturing into totally inorganic media (it is not always possible to produce a *pure* culture of autotrophic bacteria by repeated subculturing into inorganic media ; autotrophic cells die and disintegrate and so release organic compounds that can form the ' food ' of contaminating heterotrophic, i.e. ' ordinary ' bacteria). Or they may be studied in their natural habitats such as soil ; the usual technique for studies of this kind is soil percolation (Lees & Quastel, 1946 ; Lees, 1949 ; Quastel & Scholefield, 1951). This technique has been widely used in the study of chemical changes brought about by the autotrophic population of the soil ; nevertheless, for the present purposes it is a less valuable technique than the other two because it is not generally capable of yielding unequivocal answers to questions concerning the detailed biochemistry of the organisms. Therefore attention will be largely confined to results obtained by the studies of autotrophic cultures.

Autotrophic bacteria, organisms that require only a few inorganic chemicals to effect a total synthesis of all the

compounds that go to make up a living cell, obviously pose biochemical questions of the utmost interest. Yet it must be admitted that these questions remain largely unanswered. The reason is not far to seek ; autotrophic bacteria, of all organisms, are the most tedious ones to work with. Yields are always low (the maximum yield of *Nitrobacter* is about 0·02 g cells per litre of culture) and cultures are always liable to die out suddenly. It is therefore not surprising that biochemists have tended to devote their attentions to more easily available sources of living material on which research, if no less difficult, is at least more rapid. It is hoped that this book may stimulate a more practical interest in a much neglected field.

Finally, a word about the references quoted in the text. These are not meant to be exhaustive ; in particular, references to older classical work are usually omitted as they may be found in most standard text-books on bacteriology. Most of the references are deliberately confined to more modern work, particularly to biochemical work, on the autotrophic bacteria.

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THE AUTOTROPHIC WAY OF LIFE

THE etymological meaning of autotroph is 'self-nourishing'. Strictly speaking, an autotroph is an organism that can live, grow, and reproduce, in an environment free from other living organisms and free from compounds made by them. In other words, it can live, grow, and reproduce indefinitely in an inorganic environment, i.e. an environment in which the sole source of carbon is carbon dioxide*. The most common autotrophs are the green plants ; these, as is well known, may be grown in hydroponic culture where the only nutrients supplied to them, other than the carbon dioxide of the air, are the minerals added to the aerated water that is washed over the roots. Lower down in the scale of organizational complexity are the microscopical algae, which impart the green colour to stagnant pond water. These are really tiny plants, living the same type of autotrophic life as garden plants but drawing their nutrients from the water, in which they are dispersed, directly through the cell wall instead of through a specialized absorption system such as a root. Finally, there are the autotrophic bacteria, which, like the algae, draw their nutrients through the cell wall. Some, like the algae, are coloured ; some are colourless or virtually so. Some, like the algae, require light for their development, some can develop in darkness. But all, like the algae, are autotrophs capable of synthesizing every one of the complex proteins, carbohydrates, fats, and other organic compounds of which they are composed, from a carbonaceous starting material no more complex than carbon dioxide.

This implies immense synthetic ability. Autotrophic organisms, when analysed, show much the same spectrum of amino

* Or, for that matter, carbon monoxide or carbon suboxide. However, the quantitative importance of carbon dioxide as an inorganic carbon source is so overwhelming that in definitions of autotrophy other sources are usually ignored for the sake of simplicity.

acids, carbohydrates, fats, and quantitatively minor organic compounds as do heterotrophic organisms ; that is, organisms such as ourselves which require some preformed organic compound or compounds for life, growth, and reproduction. As Tracey (1954) has pointed out in his excellent survey of biochemistry, 'There are chemical likenesses between all the organisms so far studied. The most abundant sugar in a potato, a shipworm, an elephant, and a bacterium is likely to be glucose. . . . Many of the other sugars and the twenty or so amino acids that make up proteins are similarly ubiquitous.' This brings us to what is now a fundamental concept of biochemistry—that all life is based on broadly the same pattern ; that all living cells are composed of polymers of the same twenty or so amino acids (proteins), polymers of the same few hexoses (polysaccharides), triglycerides of more-or-less straight-chain fatty acids (fats), together with other small-size organic compounds, inorganic salts, and water. Although the basic units always remain the same, the polymers formed from them may be very different. The glucose polymer of animal liver, glycogen, in which chains of glucose molecules are branched like the branches of a tree, has properties very different from cellulose, the straight-chain glucose polymer characteristic of plants. Proteins (amino acid polymers) may differ in one animal as much as do hair and muscle, yet broadly speaking the amino acid composition of hair is similar to that of muscle protein ; it is in the arrangement of the amino acids that the difference between the two proteins lies.

Once this concept has been grasped, the difference between autotrophic life and heterotrophic life appears a good deal less marked. *Both* forms of life fabricate highly complicated compounds from simple ones; even so highly a heterotrophic organism as a human being requires, in theory at any rate, no more than water, mineral salts, some ten amino acids, and a few vitamins in traces, in order to live, grow, and reproduce. Even in human beings, complicated proteins and polysaccharides are always broken down to their constituent amino acids and sugars before being absorbed through the gut wall into the soma. Where autotrophs differ from heterotrophs is in their ability to fabricate, without assistance from any

5

external source of organic material, *all* the small compounds, the building stones of the cell, from carbon dioxide and mineral sources of other essential elements such as nitrogen, phosphorus, sulphur, and various metals.
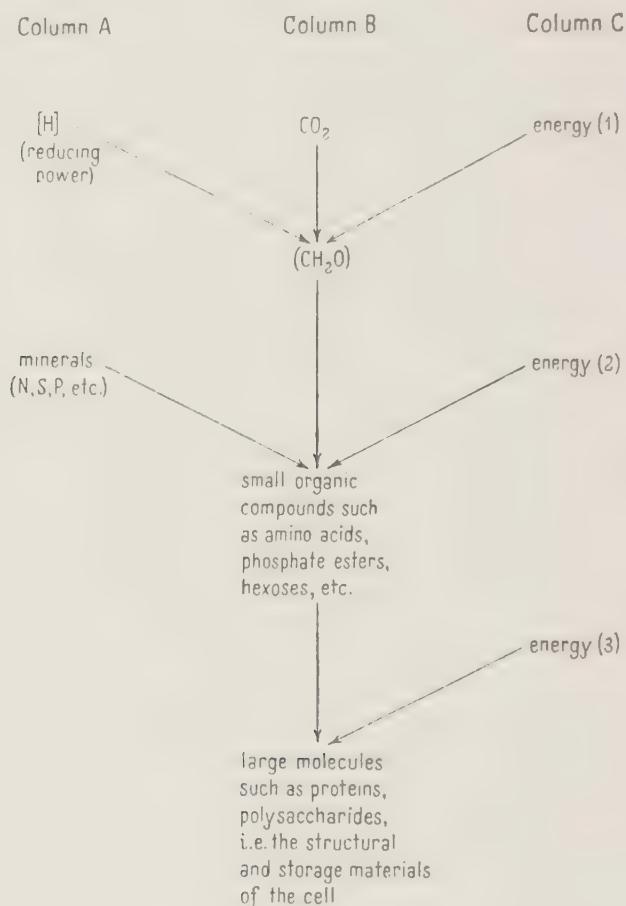
Column A   Column B   Column C

[H] CO_2 energy (1)
(reducing
power)

                       (CH_2O)

minerals   energy (2)
(N,S,P, etc.)

                       small organic
                       compounds such
                       as amino acids,
                       phosphate esters,
                       hexoses, etc.

                                              energy (3)

                       large molecules
                       such as proteins,
                       polysaccharides,
                       i.e. the structural
                       and storage materials
                       of the cell

Figure 1

But the building-up of small organic compounds from carbon dioxide will not proceed spontaneously even if all the enzymes necessary for the conversion are present. There must be some source of reducing power because the carbon in the cellular components is more reduced than it is in carbon dioxide ; and there must be some source of energy because the reduction of carbon dioxide to the ' oxidation level ' at which carbon is

6

found in the cellular components is an endergonic (energy-consuming) process. Nor will the elaboration of proteins, polysaccharides, and fats from smaller molecules proceed without some energy supply. The situation is expressed diagrammatically in *Figure 1*.

This is, of course, a highly formalized representation. The arrows do not indicate single reactions, they blanket many step reactions ; [H] does not represent hydrogen as such, but the reducing power of hydrogen carriers (e.g. DPNH, TPNH)* ; the formula CH_2O does not stand for any particular compound, it merely symbolizes the production of some carbon compound (or compounds) in which the level of oxidation of the carbon is roughly equivalent to the level of oxidation of the carbon in compounds with empirical formulae CH_2O. Nevertheless, despite its manifest sketchiness, *Figure 1* serves quite well to illustrate some of the problems connected with autotrophic bacteria.

An autotrophic bacterium may be defined as one that can live, grow, and multiply, in an environment where the sole source of carbon is carbon dioxide; it requires no preformed organic compounds for its anabolic processes; it can traverse *Figure 1* from top to bottom. A heterotrophic bacterium, on the other hand, cannot do this. At least one organic compound, perhaps some sugar or a particular amino acid, must be supplied before the bacterium can develop. There are two reasons why heterotrophic bacteria (and, for that matter, heterotrophic organisms in general) require some preformed organic compound or compounds. The compound may be an essential component in the metabolism of the bacterium, a component which, nevertheless, the bacterium cannot synthesize from the starting materials available to it. ' Growth factors ' and ' essential amino acids ' fall into this class. On the other hand, the compound may be required, not because of any peculiarity in its chemical structure, but because the bacterium can break it down and extract energy from the process, energy that can subsequently be used for anabolic

* DPNH and TPNH will be used to indicate the reduced forms of diphospho-pyridine nucleotide and triphosphopyridine nucleotide respectively. The oxidized forms will be indicated by DPN^+ and TPN^+.
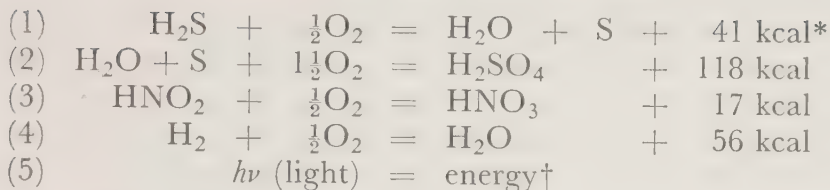
7

purposes (the energy (1), (2), and (3) of *Figure 1*). Among heterotrophic organisms the requirement for an *organic* compound to fulfil this purpose is absolute. The requirement may be quite unspecific; a single strain of a heterotrophic bacterium may be capable of growing equally well on any one of a whole range of organic compounds—in contrast to the specificity shown when an organic compound is acting as, or as a precursor to, an essential metabolite. Nevertheless, despite their general ability to use a wide range of organic compounds as sources of energy, all heterotrophs require *some* organic compound as a source of energy ; in the absence of such a source they do not grow ; in its presence their growth will be governed by the amount of energy supplied by the source. Autotrophs, on the other hand, can, by definition, live, grow, and multiply, in an environment where the only source of carbon is carbon dioxide. As carbon dioxide is not an organic compound—and cannot, in any case, be broken down to yield energy—it follows that the energy for the anabolism of autotrophs must come from sources other than the breakdown of organic compounds. In fact, autotrophic bacteria derive their energy either from light or from the energy released by inorganic reactions and may therefore be defined as ' organisms that do not use organic compounds as primary sources of energy '. This definition, which implies that heterotrophs are ' organisms that use organic compounds as primary sources of energy ', turns out to be rather more meaningful than our original ' (organisms) that can live, grow, and multiply, in an environment where the sole source of carbon is carbon dioxide ' (p. 8).

To understand why the second definition of autotrophs is more meaningful than the initial one, reconsider *Figure 1*, not as a skeletal plan subsuming the activities of all living organisms, but as a diagram drawing a distinction between autotrophs and heterotrophs. In this reconsideration traverse the diagram from top to bottom in three sections, beginning on the right-hand side.

Energy (1), (2), and (3) (Column C)

This energy, in autotrophic bacteria, derives (as we have seen) from non-organic sources. Such sources are typified by

$$
\begin{aligned}
&(1) &\qquad H_2S + \tfrac{1}{2}O_2 &= H_2O + S &&+ \quad 41 \text{ kcal*} \\
&(2) &\quad H_2O + S + 1\tfrac{1}{2}O_2 &= H_2SO_4 &&+ \quad 118 \text{ kcal} \\
&(3) &\qquad HNO_2 + \tfrac{1}{2}O_2 &= HNO_3 &&+ \quad 17 \text{ kcal} \\
&(4) &\qquad H_2 + \tfrac{1}{2}O_2 &= H_2O &&+ \quad 56 \text{ kcal} \\
&(5) &\qquad h\nu \text{ (light)} &= \text{energy†}
\end{aligned}
$$

In heterotrophic bacteria this energy derives from the breakdown of organic compounds, e.g. the oxidation of glucose to carbon dioxide and water :

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + 690 \text{ kcal}$$

Now it is well recognized that in heterotrophic organisms the energy resulting from the oxidation of glucose is not dissipated as unusable heat but is stored as the energy of high-energy bonds such as high-energy phosphate bonds ; the stored energy may then be used as an energy source for the anabolic steps (involving energy (1), energy (2), and energy (3)) shown in *Figure 1*. On the basis of general comparative biochemistry one might expect that the inorganic transformations effected by the autotrophs would similarly yield high-energy bonds capable of supplying the energy for the same anabolic steps. Unfortunately the generation of high-energy bonds has been studied in only one autotrophic bacterium (*Thiobacillus*, see Chapter 3) ; more unfortunately still, there has been considerable controversy about the validity of experimental results. However, the latest data (Umbreit, 1954) do suggest most strongly that in *Thiobacillus thio-oxidans* the oxidation of sulphur by oxygen is concomitant with the generation of organic phosphate esters having the characteristics of high-energy phosphate compounds. Whether a generation of high-energy phosphate bonds similarly accompanies the primary energy-yielding reaction (P.E.R.) of every autotrophic bacterium is not known —largely because no-one has seriously attempted to find out. It should be remembered, however, that if future experiments fail to show a generation of phosphate bond energy in autotrophs (other than *Thiobacillus*) carrying out their P.E.R., this failure would simply indicate that the accomplishment of the

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* 1 kcal = 1000 calories = $4 \cdot 2 \times 10^{10}$ ergs.
† h = Planck's constant $(6 \cdot 54 \times 10^{-27}$ ergs/sec), ν = the frequency of the light vibrations/sec. For red light, the energy is about 40 kcal per mol quantum, i.e. per 6×10^{23} light quanta.

P.E.R. did not result in the genesis of *phosphate* bond energy. It might well prove that the energy resulting from the accomplishment of the P.E.R. in autotrophs was stored as high-energy sulphur bonds and that high-energy phosphate bonds acted merely as energy carriers, of transient existence, between the energy store and the energy-consuming anabolic processes. This is speculation ; what is certain is that autotrophic bacteria can accomplish specific P.E.R.* that do not involve organic compounds, and can, moreover, transfer the energy yielded by non-organic P.E.R. to the energy-consuming, anabolic processes by which fresh cell material is elaborated from carbon dioxide.

As we are at the moment principally concerned with a comparison between autotrophs and heterotrophs, one obvious question now presents itself. Autotrophs are capable of living, growing, and proliferating, on the energy supplied by light or by some simple inorganic transformation ; heterotrophs are not. Is this because the heterotrophs are incapable of capturing light energy, incapable of carrying out the simple inorganic transformations ? Or is it because, although capable of these processes, heterotrophs cannot transfer the energy captured from light or released by an inorganic transformation, to their anabolic processes ? By and large, the second explanation seems to be the more comprehensive. Manifestly the first explanation fails in respect of the capture of light energy. Many heterotrophic bacteria, notably members of the genera *Pseudomonas* and *Xanthomonas*, are brightly coloured. They have a marked capacity for absorbing light energy, yet are quite incapable of using any of the energy captured by their pigments because, as the second explanation indicates, there is no energetic coupling between pigment system and anabolic processes. The first explanation also fails in respect of a number of inorganic transformations used by autotrophic bacteria as sources of energy because it happens that a number of such inorganic transformations, known to be used as sources of energy by various autotrophic bacteria, can be

* Each autotroph is specific as regards its P.E.R. *Nitrosomonas*, which derives its energy from the oxidation of ammonia to nitrite, is incapable of oxidizing the nitrite further. *Nitrobacter*, which derives its energy from the oxidation of nitrite to nitrate, cannot oxidize ammonia.

accomplished by certain heterotrophic bacteria although these bacteria (presumably because they lack mechanisms for the appropriate energetic coupling) cannot utilize the energy released by the transformations for anabolic purposes. Many bacteria, for instance, can oxidize molecular hydrogen ; only a few are capable of using the considerable energy released by the oxidation (p. 9, equation 4) as the sole source of energy for life, growth, and proliferation. The oxidation of ammonia to nitrite, once thought to be the exclusive domain of the autotroph *Nitrosomonas*, can be carried out, to some small extent, by a number of heterotrophic organisms (Cutler & Mukerji, 1931). More recently, Fisher, Fisher & Appleman (1952) have isolated a soil heterotroph* capable of producing nitrite in soil-extract media. Hutton & ZoBell (1953) have shown that certain methane bacteria oxidize ammonia to nitrite. According to a preliminary account by Schmidt (1954), the soil fungus *Aspergillus flavus* can produce some 16μg nitrate-nitrogen per c.c. when grown on a peptone–glucose medium ; until this account appeared, the autotroph *Nitrobacter* (Chapter 5) was thought to be the only organism capable of producing free nitrate. A number of heterotrophs share with *Thiobacillus* spp. (Chapter 3) the ability to oxidize thiosulphate to tetrathionate ; this reaction is not quite in line with the other reactions so far listed, because the energy yield is so small that not even *Thiobacillus* spp. use the reaction as a source of energy ; it is included simply to emphasize the similarity between the inorganic metabolism of heterotrophs and the inorganic meta-bolism of autotrophs. The oxidation of hydroxylamine to nitrite is carried out both by the autotroph *Nitrosomonas* (Hof-man & Lees, 1953) and by the heterotroph *Corynebacterium equi* (Lees, Simpson, Jensen & Sørensen, 1954).

Some of the reactions, then, that are used by autotrophs as primary sources of energy are also carried out by certain heterotrophs. The heterotrophs, however, do not use these reactions as primary sources of energy, either because they lack the ability to canalize the energy released into their anabolic processes, or because their anabolic processes require for their functioning carbon sources other than carbon dioxide. This

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* Tentatively designated *Diazoter*.

brings us to Column B, the column of anabolic processes, in *Figure 1*.

Anabolic processes (Column B)

The centre column of *Figure 1* shows, in a very diagrammatic way, how cell material may be built up from carbon dioxide. The absolutely essential step here is the reduction of carbon dioxide to the oxidation level (CH_2O), which is roughly the oxidation level of the carbon in the cell material as a whole; van Niel (1936) showed that, in purple photosynthetic bacteria at least, the oxidation level is more accurately represented as (C_2H_3O), but (CH_2O) will serve our present purpose well enough.

Strict autotrophs are distinguished from heterotrophs, not so much by their ability to use carbon dioxide as a source of carbon, as by their inability to use anything else. Some heterotrophic organisms, if not all, are capable of incorporating carbon dioxide into their cellular material by carboxylation reactions such as that catalysed by the malic enzyme system :

$CH_3.CO.COOH+CO_2+2[H]$
pyruvic acid $= HOOC.CH(OH).CH_2.COOH$
malic acid
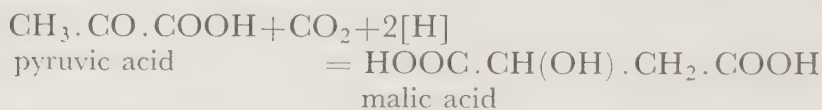
but in these organisms the ' $2[H]$ ', signifying reducing power, is ultimately obtainable only from organic sources, e.g. from lactic acid through the mediation of lactic dehydrogenase:

$CH_3.CH(OH).COOH = CH_3.CO.COOH + 2[H]$
lactic acid
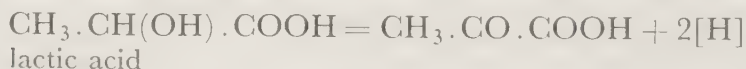
Adding these two equations, it is seen that organisms possessing the appropriate enzyme systems should be able to incorporate carbon dioxide into lactic acid to yield malic acid:

$CO_2 + CH_3.CH(OH).COOH$
$= HOOC.CH(OH).CH_2.COOH$
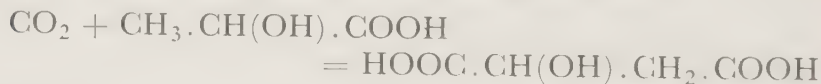
In fact, heterotrophs do incorporate carbon dioxide into their tissues in this manner, but the process is not a real *assimilation**

* By ' assimilation ' is meant the *reductive* capture of carbon dioxide by an organism, e.g. the formation of (CH_2O) from carbon dioxide. The non-reductive capture of carbon dioxide, e.g. the formation of $R.CH_2.COOH$ from $R.CH_3$, will be referred to as ' carbon dioxide fixation '.

of carbon dioxide. If carbon dioxide is to be used as source of carbon for fresh cell material, it must be reduced to the level of CH_2O. The empirical formula of lactic acid is CH_2O, that of malic acid is $CH_{1.5}O_{1.25}$. By adding carbon dioxide to the lactic acid we have moved away from the desired level of oxidation of the carbon. The capture of carbon dioxide by some such mechanism as the synergism of malic enzyme and lactic dehydrogenase could be said to result in carbon *assimilation* only if the malic acid produced were subsequently reduced to (say) succinic acid. In heterotrophic organisms this reducing power can be generated only by the oxidation of some organic compound, e.g. by the oxidation of lactic acid to pyruvic acid, and it is found in practice that the amount of organic compound that must be moved to an oxidation level higher than that of the cell constituents (to provide the necessary reducing power) is always more than the amount of carbon that is thus assimilated from carbon dioxide. In autotrophic organisms the reducing power necessary for the assimilation of carbon dioxide does not come from organic sources at all ; the autotrophs, but not the heterotrophs, are therefore true generators of fresh organic (cellular) material.

As might be expected, there are bacteria (e.g. the hydrogen bacteria) that are facultative autotrophs. In media devoid of organic matter they are able to oxidize hydrogen to water and live autotrophically on the energy released ; in ordinary ' heterotrophic ' media containing sugars, amino acids, peptone, etc., they live as normal heterotrophs. What is really more surprising is the existence of strict autotrophs, organisms that cannot live heterotrophically even in a heterotrophic medium ; most of the organisms dealt with in this book belong to that class. The nitrifying organism *Nitrosomonas*, for instance, builds up from carbon dioxide and ammonia all the common amino acids (Hofman, 1953), yet, as far as is known, it will not use any of these acids if they are supplied to it. Either the cell wall is impermeable to all carbon compounds other than carbon dioxide and the bicarbonate ion, or the metabolism of the cell is so tightly integrated that externally supplied compounds cannot be incorporated into it. The organism seems to have an absolute requirement for carbon dioxide as a carbon

13

source. Curiously enough, a requirement for carbon dioxide, although, of course, not an absolute one, is shown by many heterotrophs, especially in the early stages of their growth. Young cultures of certain heterotrophic bacteria grow either badly or not at all if carbon dioxide is not supplied (Knight, 1945). Recent work on *Clostridium kluyveri* (Tomlinson & Barker, 1954) has shown that in a medium where the carbon sources are ethanol, acetate, and carbon dioxide (this term is meant to cover free carbon dioxide, carbonate, and bicarbonate), growth of the organism is proportional to the carbon dioxide concentration up to a limit of $M/300$; growth is also proportional to the amount of carbon dioxide incorporated into the cell material. In this medium, up to 25 per cent of the cell carbon could be derived from carbon dioxide.

Growth factors

It is possible, of course, to imagine that there are heterotrophic organisms that build *all* their cell material from carbon dioxide and use organic compounds simply and solely to supply energy and reducing power for the assimilation of carbon dioxide. On the other hand, it is known that many heterotrophic organisms certainly cannot do this because some specific organic compound must be provided before the organism will grow. In this case the compound is, or is converted into, some essential metabolite that the organism cannot make for itself from the carbon sources available. Do any of the autotrophic bacteria require such 'growth factors'? If the common definition of autotrophs is used (see p. 7), the answer is, strictly speaking, No, since by that definition autotrophs are able to survive with carbon dioxide as a *sole* source of carbon. But, as Woods & Lascelles (1954) have pointed out, one can be too rigid on this issue. Some hydrogen-oxidizing bacteria, some photosynthetic bacteria, and perhaps the nitrifying bacteria too, require traces of growth factors for optimal growth ; yet any definition of autotrophs that excluded these groups of organisms (especially the first and third) would be manifestly absurd. For this reason Woods & Lascelles suggested that in the common definition of autotrophs, the concept of carbon dioxide as the *sole* source of carbon should be replaced by one

in which carbon dioxide is thought of as the *main* or *bulk* source of carbon. Of course, caution is necessary in the use of the amended definition ; the term autotroph must be reserved for organisms whose carbon source is necessarily and overwhelmingly carbon dioxide. Ultimately the decision to describe an organism as an autotroph or a heterotroph is bound to hinge on a question of sorites if classification is to be based on the amount of organic material the organism requires. From this point of view our second definition of autotrophs (p. 8), as ' organisms that do not use organic compounds as primary sources of energy ', is better since it does not exclude organisms that may require a few organic compounds as growth factors.

The reducing power [H] (Column A)

This, the remaining column of *Figure 1*, has been dealt with to some extent in the previous sections. In heterotrophs the oxidation of some organic compound generates DPNH or TPNH from DPN^+ or TPN^+ ; the DPNH or TPNH can then be used to reduce some other compound with the reappearance of DPN^+ or TPN^+. It is virtually certain that these carriers, or similar ones, operate in the autotrophic bacteria. Let us call the carrier operating A. During carbon dioxide assimilation by the autotroph, the reduced form of the carrier (AH_2) will be oxidized :

$$CO_2 + 2AH_2 = (CH_2O) + H_2O + 2A$$

The problem now is, how do the autotrophic bacteria regenerate the reduced form of the carrier ? They must do this, or carbon dioxide assimilation would cease when the initial supply of reduced carrier had been oxidized. In the photosynthetic bacteria, as in green plants and algae, only one method is possible ; the light energy must be used to split water into a reducing component and an oxidizing component, the latter is then disposed of in some way (see Chapter 8) and the reducing component used to reduce the carrier :

$$H_2O \xrightarrow[\text{energy}]{\text{light}} [H] + [OH]$$
$$2[H] + A \rightleftharpoons AH_2$$
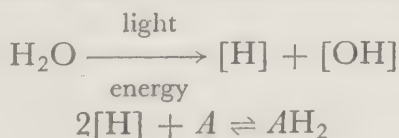
(For the benefit of those unfamiliar with the terminology of photosynthesis, it is necessary to point out that the [H] and [OH] used above merely symbolize a group with a high reducing power and a group with a high oxidizing power. *No precise formulation of these groups is implied.* There is evidence (p. 71) that, in practice, [H] may be $-$ S.H and [OH] may be $-$ S.OH, but this is not yet fully proved.) In the case of the hydrogen bacteria (Chapter 4), the ammonia-oxidizing bacteria (Chapter 5), and the non-photosynthetic sulphur bacteria oxidizing sulphide to elementary sulphur (Chapter 3), the reducing power necessary for the regeneration of AH_2 from A, *could*, in theory at any rate, be provided by the substrate itself :

$$H_2 + A \rightleftharpoons AH_2$$
$$NH_3 + 2H_2O + 3A \rightleftharpoons HNO_2 + 3AH_2$$
$$H_2S + A \rightleftharpoons S + AH_2$$
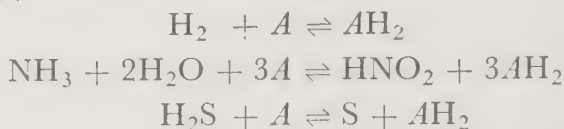
always assuming that suitable enzyme systems are present. Even where the substrate does not contain hydrogen, as in the case of *Nitrobacter* oxidizing nitrite to nitrate, one can assume that a hydrated form of the substrate is involved :

$$H_2O . NO_2^- + A \rightleftharpoons AH_2 + NO_3^-$$

Such formulations are simple and attractive ; their drawback is, however, that they represent reversible equilibria, and it might be, in practice, that if the reaction were to proceed appreciably to the right-hand side, the concentration of AH_2 would have to be maintained at an impossibly low value. The nitrate reductase of *Neurospora*, for instance, catalyses the reaction

$$TPNH + H^+ + NO_3^- \rightleftharpoons TPN^+ + NO_2^- + H_2O$$
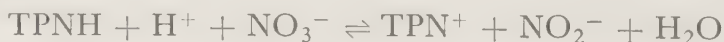
but, as Nason & Evans (1953) showed, the equilibrium is entirely on the side of nitrite production, since the rH* of the $TPN^+ - TPNH$ system is about $4 \cdot 0$, while that of the $NO_2^- - NO_3^-$ system is about 30 at pH 7.

* For any redox system, the rH is the negative logarithm of the (perhaps theoretical) hydrogen pressure in equilibrium with it. This, in the writer's opinion, is a far more useful way of characterizing a redox system than the E'o nomenclature (see *Multi-enzyme Systems*, by Malcolm Dixon, Cambridge Univ. Pr., 1949).

An alternative theory is that the substrate is oxidized at the expense of some cytochrome* system, e.g.

$$NO_2^- + H_2O + 2Cyt^{+++} = NO_3^- + 2H^+ + 2Cyt^{++}$$
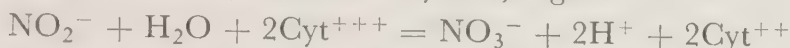
and that phosphate bond energy (or some other form of chemically stored energy) is generated by the reoxidation of the cytochrome to the higher-valency form by molecular oxygen :

$$2Cyt^{++} + 2H^+ + \tfrac{1}{2}O_2 = H_2O + 2Cyt^{+++} + \text{energy}$$

Finally, the (bond) energy produced may be used to split water into an oxidizing component and a reducing component as light energy is used in photosynthetic organisms, although this does not imply that the [H] and [OH] produced when water is split by chemical bond energy are necessarily identical with the [H] and [OH] produced by light energy. Nevertheless, we may suppose that the [OH] is disposed of and the [H] used to regenerate AH_2 from A exactly as in photosynthetic organisms ; regeneration of AH_2 will certainly occur if the rH of the [H] is low enough and suitable coupling mechanisms are present.

Both theories are, however, speculative. They are merely suggestions *how* reducing power might be generated by an inorganic oxidation and transferred to carbon-dioxide assimilating systems within the framework of present biochemical knowledge. It may be that neither is correct; it may be that one type of transfer is found in one autotroph and a different type in another. The only satisfactory answer lies in further research on the autotrophic bacteria themselves.

The remaining item in the column, the minerals, can be dealt with very briefly. Except in so far as they may be part of the structure of (possibly required) growth factors, such as the sulphur of biotin, all mineral elements can be supplied to the autotrophs as inorganic salts ; sulphur as sulphate, phosphorus as phosphate, etc. Some organisms may, however, prefer their nitrogen to be supplied as ammonia rather than as nitrate, others may prefer nitrate ; nitrite is usually unacceptable

* By ' cytochrome ' is meant any terminal oxidation system incorporating a metal or metals capable of existing in two valency states and also capable of generating high-energy bonds on transition from the lower to the higher valency state.

and often toxic at anything except very low concentrations. Trace elements such as iron, copper, molybdenum, zinc, cobalt, manganese, etc., may also be required in small amounts since they are often components of essential enzyme systems. These remarks are meant of course to apply only to the mineral *nutritional* requirements of the autotrophs ; where an autotroph uses an inorganic compound in its P.E.R., the requirement will be quantitatively much greater and qualitatively more exacting (see footnote, p. 10).

We have now completed our survey of *Figure 1*. The distinction between autotrophs and heterotrophs, if it seemed clear at first, has become rather blurred. Some autotrophic bacteria require growth factors and therefore cannot, strictly speaking, be said to be capable of flourishing in a medium in which the sole source of carbon is carbon dioxide. Some heterotrophic bacteria have a genuine requirement for a certain amount of carbon dioxide, a requirement that cannot, apparently, be satisfied by a preformed organic compound. Because carbon dioxide is an important metabolite to both autotrophs and heterotrophs (supremely so to the former, and sometimes appreciably so to the latter), an attempt was made (p. 8) to define autotrophs as ' organisms that do not use organic compounds as primary sources of energy '. This definition might fail on the trivial objection that there are facultative autotrophs that can, when living heterotrophically, use organic compounds as sources of energy, but the objection can be met by reserving the definition to their autotrophic mode. A more powerful objection to the definition is to be found in the metabolism of the Athiorhodaceae (Chapter 7). These organisms derive their energy from light when grown in the light, but they also require organic compounds in order to absorb the oxidizing component produced by the photolysis of water (p. 15). The total metabolism is thus that carbon dioxide is reduced and an organic compound oxidized, but *all* the energy for the carbon dioxide assimilation comes from light. Are these autotrophs or are they not ? Some would say ' yes ', others ' no '. Perhaps the best definition is, ' it is an autotroph if the experts on autotrophs say it is ', with the proviso that ' experts often differ ' !

The free-energy efficiency of the autotrophs

It is possible to calculate, with reasonable precision, how much energy is made available to an autotrophic bacterium by an inorganic transformation or the capture of a photon. For instance, the oxidation of nitrite to nitrate by molecular oxygen yields about 17 kcal/g atom of nitrogen. It is also known that the reduction of carbon dioxide to the level CH_2O (which we can assume is the level in the cell) will consume about 120 kcal/g atom of carbon. If observations are made on the amount of carbon incorporated into the cell material of *Nitrobacter* during the oxidation of a known amount of nitrite by this organism, we can calculate how efficiently energy is transferred from the nitrite oxidation to the carbon dioxide reduction processes. We can then express the free-energy efficiency of *Nitrobacter* as

$$\frac{\text{Atoms of carbon assimilated}}{\text{Molecules of nitrite oxidized}} \times \frac{120}{17} \times 100 \text{ per cent}$$

This calculation was carried out, on figures published by other workers, for all the chemosynthetic* autotrophs by Baas-Becking & Parks (1927). With the exception of the hydrogen-oxidizing bacteria, the chemosynthetic autotrophs proved to be rather inefficient organisms with free-energy efficiencies of about 7 per cent. This should be regarded as a minimal figure. The calculations were based on the amount of organic carbon found in rather old cultures of autotrophs ; this amount of carbon is not the total amount assimilated, but the amount assimilated *minus* the amount lost again as carbon dioxide during respiration. All living cells are 'open systems',† essentially unstable and constantly disintegrating. This disintegration must be balanced by equally constant repair processes which require energy as well as fresh building material. Moreover, autotrophs producing poisonous end-products such as nitrite or sulphuric acid may be compelled to expend some energy in preventing these end-products from diffusing appreciably into the cell interior. An autotroph may therefore consume energy, and effect a turnover of carbon dioxide, without

* Autotrophs which gain their energy from an inorganic transformation, as opposed to ' photosynthetic ' autotrophs which use light energy.
† For a note on ' open systems ', *see* references at the end of this chapter.

growing at all, but this does not mean that it is 'inefficient' in the sense that much energy is lost during its transfer from the P.E.R. to the carbon-dioxide assimilating systems. It is this transfer efficiency, the 'intrinsic' efficiency, that seems, to the writer at least, to be biochemically more interesting. It might be expected that, in short-term experiments with young cultures, the 'overall' efficiency, the efficiency measured by Baas-Becking & Parks, would approach the 'intrinsic' efficiency since factors leading to a low overall figure—the accumulation of toxic products, respiration losses—would here be minimal. Such modern determinations as have been carried out by short-term experiments do in fact suggest that the chemosynthetic autotrophs are indeed more efficient than had been supposed. This point will be dealt with in the subsequent chapters, where we shall consider the different types of autotroph individually.

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NOTE. On p. 19, living cells are referred to as 'open systems'. Open systems are not wholly amenable to treatment by the classical methods of thermodynamics. As a consequence, in recent years, a new subject 'Irreversible Thermodynamics' or the 'Thermodynamics of Open Systems' has been evolved. An excellent introduction to this fascinating subject is given by R. O. Davies (*Penguin Science News*, **28**, 1953).

THE NON-PHOTOSYNTHETIC SULPHUR BACTERIA

A NUMBER of different bacteria can live autotrophically on the energy released by the oxidation of elementary sulphur or inorganic sulphur compounds. They are usually divided into two groups, those that deposit sulphur inside the cell and those that may, under certain conditions, deposit sulphur outside the cell.

Organisms depositing sulphur inside the cell

The morphology of these organisms is very varied ; at least two distinct families, the Beggiatoaceae and the Achromatiaceae, are included in the group. A discussion of the different morphological types involved is quite outside the scope of this book ; a clearly presented survey of the complex morphology of the organisms is given by Bisset & Grace (1954).

One of these organisms, *Beggiatoa*, was the organism on which Winogradsky (1887) originally based his concept of chemosynthetic autotrophs. *Beggiatoa* was found to require carbon dioxide for growth, and to oxidize sulphides to sulphur, the the sulphur being deposited as granules within the cell. When supplies of sulphide were exhausted, the intracellular sulphur was oxidized to sulphate. When all the sulphur had been oxidized, the organism died. Later work by Kiel (1912) on pure cultures of *Beggiatoa* and the related *Thiothrix* (Winogradsky did not have pure cultures) fully established their autotrophic character since the organisms were able to develop in the absence of organic material. The oxidation reactions are :

$$H_2S + \tfrac{1}{2}O_2 = S + H_2O + 41 \text{ kcal}$$
$$S + 1\tfrac{1}{2}O_2 + H_2O = H_2SO_4 + 118 \text{ kcal}$$
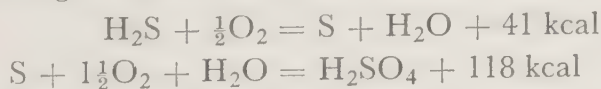
The organisms have been little studied biochemically ; most of the biochemical work on the sulphur bacteria has been carried out on the second group.

Organisms that may deposit sulphur outside the cell

These are all included in the genus *Thiobacillus*. Small, Gram-negative, rod-shaped cells, usually motile by a single polar flagellum. Strict autotrophs.

Three species are well recognized: *T. thio-oxidans*, *T. thioparus*, and *T. denitrificans*. The first two are strict aerobes capable of oxidizing a variety of inorganic sulphur compounds and growing autotrophically on the energy released by the oxidation. *T. denitrificans* is, however, a facultative anaerobe; aerobically, it behaves as the first two, anaerobically it can oxidize inorganic sulphur compounds in the presence of nitrate. The nitrate, which acts as an oxygen donor, is converted to nitrogen as the sulphur compounds are oxidized; *T. denitrificans* can grow autotrophically on the energy released by this 'anaerobic oxidation'. *T. thio-oxidans* is distinguished from *T. thioparus* by differences in acid tolerance. Some strains of *T. thio-oxidans* are still active at pH 0, while *T. thioparus* is active only in neutral media. This may be a distinction without a difference. Baalsrud & Baalsrud (1952) were able to culture six strains of thiobacilli with acid tolerances ranging from pH 5·2 to pH 1·5. Baalsrud (1954) has therefore, very reasonably, suggested that *T. thioparus* and *T. thio-oxidans* should be regarded as 'two extremes among a group of similar organisms rather than as two distinct species'.

The oxidation of sulphur and inorganic sulphur compounds by thiobacilli

The 'ideal' thiobacillus is able to oxidize sulphide, elementary sulphur, thiosulphate, or tetrathionate, to sulphate. The word 'ideal' is used because not all strains of thiobacilli are able to carry out all these oxidations, or at least are not able to do so when the different substrates are supplied extracellularly (see p. 24). However, if this complication is disregarded for the moment, it may be postulated that, in the ideal thiobacillus, the oxidation path of sulphide is

$$SH^- \xrightarrow{\text{(1)}} S \xrightarrow{\text{(2)}} S_2O_3^{--} \xrightarrow{\text{(3)}} S_4O_6^{--} \xrightarrow{\text{(4)}} SO_4^{--}$$

sulphide   sulphur   thiosulphate   tetrathionate   sulphate
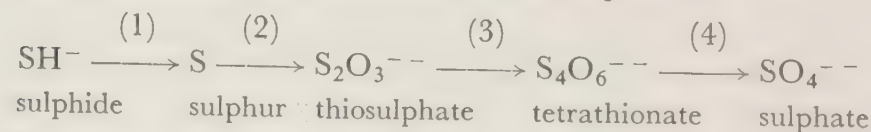
The approximate energy yields of the different oxidation steps,

22

when oxygen is the oxidizing agent, are, per atom of sulphur oxidized : (1) 40 kcal, (2) 15 kcal, (3) 5 kcal, (4) 100 kcal. The low energy yield of reaction (3), the conversion of thio-sulphate to tetrathionate, suggests that this reaction would not contribute significantly to the energy required for growth. Baalsrud & Baalsrud (1954), working with *T. denitrificans*, concluded that the reaction did not supply appreciable amounts of energy to the cell. An enzyme catalysing the reaction, tetrathionase, is found in a number of heterotrophic bacteria. At one time it was thought that these organisms were facul-tatively autotrophic and could live chemosynthetically on the energy yielded by the reaction. This view is now believed to be incorrect.

Little is known about the reaction mechanisms of reactions (1) and (2). The reverse reaction, i.e. the production of sulphide from various sulphur compounds, including thio-sulphate, has been studied in a number of heterotrophic bacteria by Olitzki (1954). He suggested that pyridoxal phosphate was involved in sulphide production ; the addition of pyridoxal phosphate to organisms whose sulphide-producing powers had been impaired by penicillin restored their activity. Whether sulphide oxidation in thiobacilli involves similar mechanisms remains to be seen. Youatt (1954) has made the interesting suggestion that *T. thiocyanoxidans* (see p. 27) oxidizes sulphide to thiosulphate *via* thiosulphite ($S_2O_2^{--}$) rather than through sulphur.

Vishniac (1952) has investigated reactions (3) and (4) in *T. thioparus*. Working with the Warburg apparatus he found that the initial rate of oxygen uptake by a *T. thioparus* suspen-sion oxidizing thiosulphate was about 30 µl./min. After some five minutes the rate dropped sharply to 2 µl./min. The total oxygen consumption at this point was equal to that required for complete oxidation of thiosulphate to tetrathionate, and the subsequent oxidation rate of 2 µl./min agreed closely with that initially observed in a companion Warburg vessel con-taining a similar amount of *T. thioparus* suspension but supplied with tetrathionate in place of thiosulphate. After a further 45 minutes, the oxygen uptake rate for both vessels dropped to about 1 µl./min ; this corresponded with the initial rate

displayed by a companion vessel supplied with trithionate $(S_3O_6^{--})$ in place of thiosulphate or tetrathionate. Vishniac concluded, therefore, that *T. thioparus* oxidized thiosulphate to tetrathionate and tetrathionate to trithionate. Difficulties in preparing higher polythionates prevented further investigation of the oxidation path eventually leading to sulphate. He was, however, able to show that the transitory formation of elementary sulphur in cultures of *T. thioparus* growing on thiosulphate was due, in all probability, to simple chemical reaction between polythionates formed in the medium by biological action :

$$2S_4O_6^{--} = S_5O_6^{--} + S_3O_6^{--}$$
$$S_5O_6^{--} = S_4O_6^{--} + S$$
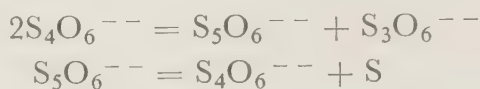
and not to specific biological formation of elementary sulphur, as had sometimes been suggested.

With this explanation of sulphur formation, we virtually reach the limit of our present knowledge about the biological and chemical reactions involving sulphur transformations in thiobacillus cultures. That this knowledge is scanty is obvious enough. Its scantiness is emphasized by the fact that what has been presented is a simplified and idealized account drawn from a number of different experimental findings. In practice, different strains of thiobacilli, possibly because of chemical reactions between the sulphur compounds they produce in the medium, possibly because one enzyme is a little more active in some strains than in others, do show marked differences in their metabolism of different sulphur compounds. Our inability to explain these differences emphasizes our ignorance.

Parker & Prisk (1953) isolated seven strains of thiobacilli from corroded concrete*. They tested the abilities of these strains to oxidize hydrogen sulphide, sulphur, thiosulphate, and tetrathionate. Thiosulphate was oxidized by all strains; three converted it to sulphate and tetrathionate, subsequently oxidizing the tetrathionate to sulphate. One strain converted thiosulphate to sulphate and sulphur, and then partially

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* By producing sulphuric acid from less acidic sulphur compounds, the thiobacilli do great damage to concrete, stone, and other structures especially in cities. See, for instance, the review by Postgate (1954).

oxidized the sulphur to sulphate. The remaining strains oxidized thiosulphate to sulphate and tetrathionate. Only four strains oxidized sulphur, from which they all produced sulphate. Hydrogen sulphide was oxidized by only two strains ; these produced sulphate from it. However, the hydrogen sulphide was supplied at the fairly high rate of 200 ppm in the air used to aerate the cultures, and at this concentration it may well have been poisonous to organisms that would have been able to oxidize it had it been supplied in lower concentration ; it would be unwise to conclude that the other five strains were *incapable* of oxidizing hydrogen sulphide. The most unexpected finding was, however, that only one strain could oxidize tetrathionate when tetrathionate was supplied as the sole source of sulphur. If this finding is confirmed it shows either that different strains of thiobacilli differ fundamentally in their metabolism of sulphur compounds or that tetrathionate oxidation by thiobacilli is dependent upon the existence of certain conditions, within or without the cell, fulfilled in the experiments of certain other workers but not in the experiments of Parker & Prisk. Present knowledge of sulphur metabolism in the thiobacilli is so meagre that it would be unwise to reject either alternative as unacceptable. Indeed, almost the only clue as to the type of enzymes involved in sulphur metabolism by thiobacilli is provided by the work of Vogler, LePage & Umbreit (1942), which showed that sulphur oxidation by *T. thio-oxidans* was inhibited by various compounds (cyanide, azide, carbon monoxide) known to inhibit cytochrome systems.

Thiobacillus thiocyanoxidans

In 1934, Happold & Key found in gasworks effluents an organism which, they believed, was an autotroph that obtained its energy from the oxidation of thiocyanate to sulphate, carbonate, and ammonia. The organism, *T. thiocyanoxidans*, was obtained in pure culture in 1951 (Happold, Johnstone & Rogers, 1952) and re-isolated in 1953 (Happold, Johnstone, Rogers & Youatt, 1954). Morphologically it proved to be a round-ended rod, $0·5\mu$–$1·5\mu$ long and about $0·3\mu$ wide, motile by a single polar flagellum. Its cultural characteristics were those of an autotroph as it could be grown in a

neutral medium consisting simply of phosphate and thio-cyanate. No growth took place on organic media unless thiocyanate was present. A number of organic compounds (peptone, yeast extract, glutamate, lactate, pyruvate, acetate, citrate, fumarate, succinate, glucose, and phenol) proved to be inhibitory to growth at $1 \cdot 0$ per cent concentration. No stimulation of growth by organic compounds was observed. Growth also took place in autotrophic media if the thiocyanate was replaced by sulphur or thiosulphate. It was necessary to supply ammonia and carbon dioxide if the organism were grown on thiosulphate or sulphur but not if it were grown on thiocyanate.

The overall oxidation of thiocyanate carried out by *T. thiocyanoxidans* is apparently

$$CNS^- + 2O_2 + 2H_2O = SO_4^{--} + NH_4^+ + CO_2 + 220 \text{ kcal}$$
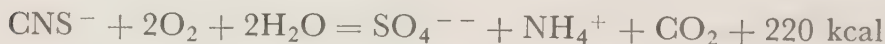
Youatt (1954) has investigated the mechanism of this oxidation. Her results suggest that the first step is a hydrolysis of the thiocyanate into sulphide and cyanate :

$$CNS^- + H_2O = HCNO + SH^-$$
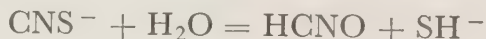
followed by a hydrolysis of the cyanate into ammonia and carbon dioxide :

$$HCNO + H_2O = CO_2 + NH_3$$
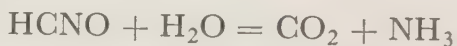
The sulphide formed in the first hydrolysis is then oxidized to sulphate as it would be in an 'ordinary' thiobacillus, thus providing energy for the growth of the organism. The carbon and nitrogen required for growth are provided by the products of the second hydrolysis. When the organism is grown on sulphur or thiosulphate, these products, carbon dioxide and ammonia, must, as we have seen, be provided separately.

Experiments showed that the organism could oxidize sulphide to sulphate as long as the sulphide concentrations were below $M/600$; above this concentration the sulphide began to have a toxic effect. However, it was possible to follow sulphide oxidation by providing the organisms with thioacetamide or thioacetate ; these compounds hydrolysed slightly in solution to sulphide, which was oxidized by the organism to sulphate.

The removal of the sulphide by oxidation disturbed the hydro-lysis equilibrium and further hydrolysis of the thioacetamide or thioacetate took place, yielding more sulphide. These com-pounds, in effect, acted as large stores of sulphide at a low concentration.

Cells *grown* in thiosulphate media oxidized thiocyanate rather slowly when washed suspensions were studied in the Warburg apparatus, but the speed of oxidation increased as the experiment progressed ; in contrast, thiosulphate was immediately and rapidly oxidized. Cells grown in thio-cyanate media showed immediate and rapid oxidation of both thiocyanate and thiosulphate when studied by the same tech-nique. These results suggest that thiocyanate oxidation is to some extent an adaptive process in the organism, whereas thiosulphate oxidation is constitutive, as it is in 'ordinary' thiobacilli. Tetrathionate was oxidized only slowly, if at all, by washed suspensions of cells. This is further evidence that tetrathionate may not be an invariable intermediate in thio-sulphate oxidation, and it led Youatt to propose a scheme of oxidation not involving polythionates :

$$SH^- \to S_2O_2^{--} \to S_2O_3^{--} \to S_2O_5^{--} \to S_2O_7^{--} \to SO_4^{--}$$
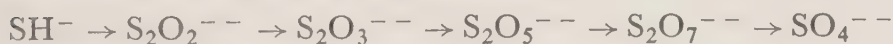
This is an interesting scheme, but clearly a great deal of work would be necessary to prove or disprove it.

Thiobacillus ferro-oxidans

There have been many reports of autotrophic bacteria capable of living on the energy released by the oxidation of ferrous iron to ferric :

$$Fe^{++} = Fe^{+++} + e + 11 \cdot 3 \text{ kcal}$$
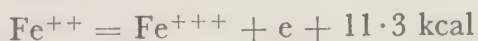
Whether the reported autotrophs were really autotrophs has always been doubtful ; moreover, their culture usually demanded an approximately neutral medium, in which the abiological oxidation of iron could take place readily under aerobic conditions. However, Temple & Colmer (1951) have reported what is apparently a genuine autotrophic bacterium capable of oxidizing ferrous iron to ferric at pH 2 ; at this pH abiological oxidation of the iron is virtually zero. The

organism was isolated from a bituminous-coal drainage water. It grew when cultured in a mineral medium containing ferrous iron and concomitantly oxidized the iron to the ferric state. No oxidation of iron took place in sterilized cultures, nor in live cultures in the absence of carbon dioxide. Microscopically it was indistinguishable from *T. thio-oxidans* and could, indeed, be cultured on a thiosulphate medium as well as on a ferrous iron medium. It is therefore probably a member of the thiobacilli possessing the peculiar extra ability of oxidizing iron, much as *T. thiocyanoxidans* has the extra ability of hydrolysing thiocyanate. It has been named *T. ferro-oxidans*. Details of its sulphur metabolism have not yet been published.

Thiobacillus denitrificans

This organism, which has been very thoroughly studied recently by Baalsrud & Baalsrud (1954), differs from the other thiobacilli, not in its oxidation of sulphur and inorganic sulphur compounds, but in its ability to use nitrate instead of oxygen as the oxidizing agent. When cultured under anaerobic conditions, it oxidizes sulphur or inorganic sulphur compounds to sulphate with the concomitant reduction of nitrate to nitrogen :

$$2NO_3^- + H_2O = 2OH^- + N_2 + 5[O]$$

$$\searrow$$
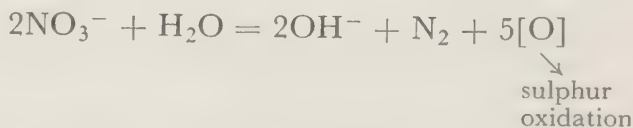
sulphur
oxidation

The hydroxyl ions produced by the nitrate reduction tend to neutralize the hydrogen ions produced by the oxidation of the sulphur or sulphur compounds ; therefore, under anaerobic conditions, with nitrate as the oxidizing agent, the pH of growing cultures of *T. denitrificans* does not drop so sharply as in aerobic cultures of thiobacilli growing on the same sulphur compound. *T. denitrificans* grows most readily at neutral reaction ; in this it resembles *T. thioparus*. *T. denitrificans* will also grow aerobically in the same way as the other thiobacilli ; under these conditions it does not reduce nitrate to nitrogen but uses oxygen as the oxidizing agent in a perfectly normal manner. If grown aerobically for any length of time it tends to lose its ability to reduce nitrate under anaerobic conditions.

It may be grown satisfactorily with either sulphur or thio-sulphate as the oxidizable compound. If it is grown under anaerobic conditions in a nitrate-thiosulphate medium in which the molar ratio of nitrate/thiosulphate exceeds about 1·25, nitrite, which represents the first stage of nitrate reduction, accumulates in the medium and poisons the cells. Baalsrud & Baalsrud suggest that unsuspected nitrite accumulation may have been the reason why other workers in the past had often found difficulty in growing *T. denitrificans*, and to obviate this difficulty they suggest that the molar ratio of nitrate/thio-sulphate in media used for culture of *T. denitrificans* should not exceed 0·4. Curiously enough, although nitrite must always be produced, albeit transiently, by *T. denitrificans* growing anaerobically, the organism is much more readily poisoned by nitrite than is *T. thioparus*, which it so closely resembles in many ways.

Warburg experiments on washed suspensions of *T. denitrificans* showed that sulphur oxidation is inhibited by nitrite while thiosulphate oxidation is not. Indeed, in the presence of nitrite, thiosulphate is oxidized very rapidly while the nitrite is oxidized to nitric oxide ; the evolution of nitric oxide can be observed manometrically, and, since no evolution takes place if the cells are poisoned with mercuric chloride, it may be concluded that the action of thiosulphate in promoting nitrite reduction is a biological and not a chemical one. The nitric oxide is then further reduced to nitrogen by the cells, presum-ably through nitrous oxide, which can also be reduced by the organisms. When tetrathionate and nitrite are added to the cell suspension, no gas evolution takes place, from which it follows that thiosulphate oxidation is probably coupled in an obligatory way with nitrite reduction :

$$2Na_2S_2O_3 + 2NaNO_2 + 2H_2O = Na_2S_4O_6 + 2NO + 4NaOH$$
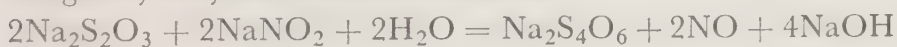
Energy transfer in the thiobacilli

In 1942, Vogler & Umbreit presented results that suggested that *T. thio-oxidans* oxidizing sulphur generated high-energy phosphate bonds and that the energy so stored could be used to effect the assimilation of carbon dioxide even after sulphur oxidation had reached completion. They found that

when *T. thio-oxidans* oxidized sulphur in the absence of carbon dioxide, there was a diminution of inorganic phosphate within the cell and a parallel increase in organic phosphate. When carbon dioxide was subsequently admitted, there was an up-take of carbon dioxide by the cells with a simultaneous release of inorganic phosphate from the organic phosphate fraction of the cell phosphorus. Qualitatively, these results were acceptable ; quantitatively, they were doubtful. *Inter alia* they implied that the energy from the sulphur oxidation was transformed into phosphate bond energy and then used to provide energy for carbon-dioxide fixation with an overall efficiency of almost 100 per cent. To say the least, such a high efficiency is doubtful, but, as Umbreit (1954) has pointed out, techniques available twelve years ago are not comparable with those available now. It would be foolish to reject out of hand results of more than a decade ago simply because their quantitative basis is, in the light of modern results by modern techniques, somewhat doubtful.

Against the results of Vogler & Umbreit, those of Baalsrud & Baalsrud (1952, 1954) must be set. These workers failed to find any residual carbon-dioxide assimilating power in thiobacilli that had oxidized thiosulphate in the absence of carbon dioxide. Such organisms, when subsequently exposed to carbon dioxide, did not assimilate any of it. Only when thiosulphate oxidation was concomitant with the presence of carbon dioxide did any assimilation of carbon dioxide occur. Baalsrud & Baalsrud showed, incidentally, that carbon dioxide absorbed by the cells during thiosulphate oxidation was genuinely *assimilated* by reductive processes and not merely absorbed by a reversible carboxylation reaction. The carbon dioxide was taken up by an overall reaction

$$CO_2 + H_2O + \text{energy} = (CH_2O) + O_2$$
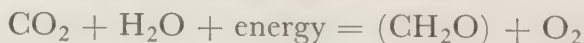
and not by an overall reaction such as

$$R.CH_3 + CO_2 = R.CH_2.COOH$$

since the oxygen uptake of cells oxidizing thiosulphate in the presence of carbon dioxide was less than that of cells oxidizing thiosulphate in the absence of carbon dioxide by a factor closely corresponding to the amount of carbon dioxide taken up by the

cells during the thiosulphate oxidation. Vogler & Umbreit had not made any such demonstration in their 'delayed carbon dioxide fixation' experiments, although they had done so in experiments where carbon dioxide was present during the oxidation of the sulphur. Nor could Baalsrud & Baalsrud find any storage of phosphate bond energy. Cells oxidizing thiosulphate took up inorganic phosphate from the medium, but this was always released again into the medium when thiosulphate oxidation was complete irrespective of whether carbon dioxide had been admitted to the system or not.

Umbreit (1954) has now answered these criticisms. He has pointed out that Baalsrud & Baalsrud used thiosulphate oxidation, and not the sulphur oxidation used by Vogler & Umbreit. Moreover, he has repeated the original experiments himself (Dr Vogler was killed in the war), but with radioactive phosphorus and radioactive carbon in the phosphate and carbon dioxide. The results of his experiments seem to confirm that (a) some energetic system capable of assimilating carbon dioxide is stored in the cells of *T. thio-oxidans* that have oxidized sulphur in the absence of carbon dioxide, and (b) high-energy phosphate bonds are stored within the cells during sulphur oxidation ; they disappear rapidly when carbon dioxide is admitted and their spontaneous disappearance in the absence of carbon dioxide is slow. Umbreit stresses that the changes of phosphate distribution he has measured are those taking place *within* the cells, which, as in the original experiments, were lysed with alkali prior to analysis. Baalsrud & Baalsrud used trichloracetic acid as a cell extractant ; according to Umbreit, no appreciable amount of phosphate is extracted by this reagent. Moreover, Umbreit has also established that the *amount* of carbon-dioxide assimilating power stored within cells that have oxidized sulphur in the absence of carbon dioxide is proportional to the amount of sulphur oxidized. This seems strong evidence in favour of the 'energy storage' theory.

Newburgh (1954) has also reinvestigated the problem, independently of Umbreit. He found that cells that had oxidized sulphur retained a certain amount of carbon-dioxide assimilating power under anaerobic conditions, but that this power was much greater in cells that had oxidized sulphur in the air

(0·03 per cent CO_2) than in cells that had oxidized sulphur in the complete absence of carbon dioxide.

The situation is thus rather confused. There seems to be little doubt that thiobacilli can store carbon-dioxide assimilating power, but for reasons not understood they do so only under certain experimental conditions.

The free-energy efficiencies of the thiobacilli

The calculations of Baas-Becking & Parks (1927) put the free-energy efficiency of the thiobacilli between 5 and 9 per cent. There are reasons (p. 19) for supposing that this ' overall ' efficiency might be exceeded if the experimental period were shortened. This is possible in the Warburg apparatus, where the amount of substrate oxidized and the amount of carbon dioxide assimilated may both be measured manometrically over an experimental period of a few hours. The Warburg data of Vogler (1942) yield a maximum short-term efficiency for *T. thio-oxidans* of about 50 per cent ; the data of Baalsrud & Baalsrud (1952) yield maxima of 16 per cent for the aerobic thiobacilli and 25 per cent for *T. denitrificans*. Youatt (1954) found that one molecule of carbon dioxide was assimilated for each two thiocyanate ions oxidized by *T. thiocyanoxidans* ; since each thiocyanate ion releases some 220 kcal on oxidation, this organism, too, shows an efficiency of about 25 per cent. Temple & Colmer (1951) give an efficiency of some 3 per cent for *T. ferro-oxidans* oxidizing ferrous iron. Here, however, the reaction of the external medium (pH 2) is so unfavourable that the cells must waste a considerable amount of energy merely in maintaining a reasonable internal pH against a relatively enormous pressure of hydrogen ions. Moreover, the energy-yielding reaction itself is so feeble that one single high-energy phosphate bond can only just be generated per electron produced, even if the generating mechanisms are 100 per cent efficient. A low efficiency for this organism carrying out ferrous iron oxidation is therefore not surprising.

Composition of the thiobacilli

One member of the thiobacilli, *T. thio-oxidans*, has been more thoroughly analysed than any other autotrophic bacterium.

It contains all the common amino acids (Frantz, Feigelman, Werner & Smythe, 1952). A variety of phosphorus-containing compounds are present (LePage & Umbreit, 1943), including ATP, hexose diphosphate, phosphoglyceric acid, fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate, and DPN ; *in toto* about 90 per cent of the acid-soluble phosphorus of the cell is accounted for by these compounds. It is said that the ATP is present as adenosine-3′-triphosphate instead of the normal adenosine-5′-triphosphate. Storage polysaccharide is present (Knaysi, 1943) and is metabolized to provide energy for respiration when the cells are starved of oxidizable sulphur compounds (LePage, 1942). Indeed, the composition of *T. thio-oxidans* is much the same as that of any ordinary heterotrophic bacterium ; the only unusual compound found in it so far being the peculiarly constituted ATP. It is an excellent example of how the same biochemical pattern persists in all forms of life, no matter how extraordinary they may appear to be at first sight.

Nutrient requirements of the thiobacilli

It seems likely that only ammonium salts can serve as a source of nutritional nitrogen for the thiobacilli, nitrate will not do ; this applies even to *T. denitrificans* (Baalsrud, 1954). Manganese and iron are essential, the latter presumably being needed for, *inter alia*, cytochrome systems. Cytochrome bands (at 548 mμ and 522 mμ) were detected in *T. denitrificans*, although, curiously enough, not in the aerobic species (Baalsrud & Baalsrud, 1954). However, they were detected in such species by Emoto (1933). Vishniac (1949) found that he obtained better yields of *T. thioparus* when he added succinate to the mineral medium, but the increase in yield was independent of the concentration of the succinate used ; perhaps the succinate was acting as a growth factor in the sense that endogenous formation of succinate within the cell was so slow in the mineral medium as to be the process ultimately limiting the growth rate of the cells. This finding emphasizes the difficulty of defining an autotroph, as does the discovery by Youatt (1954) that *T. thiocyanoxidans* will oxidize formate. Yet no-one doubts that the thiobacilli are autotrophs. Rittenberg & Grady (1950) produced, by u.v. irradiation, a mutant of

T. thio-oxidans that required thiamin for growth, thus showing that thiamin is as essential to the internal economy of *T. thio-oxidans* as it is to that of heterotrophic organisms—another example of the unity of biochemistry.

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THE HYDROGEN BACTERIA

MANY bacteria, of diverse morphological types, are able to live autotrophically on the energy released by the oxidation of hydrogen gas :

$$H_2 + \tfrac{1}{2}O_2 = H_2O + 56 \text{ kcal}$$

None of these organisms is a strict autotroph : all can live heterotrophically on ordinary organic media. Strict hetero-trophs are known that can oxidize hydrogen gas but cannot use the energy released for growth purposes. The hydrogen bacteria, which can live either autotrophically by aerobic oxidation of hydrogen, or heterotrophically, are thus one of the most patent links between the autotrophic and heterotrophic ways of life. They are sometimes known by their German title of ' Knallgasbakterien '.*

The best studied of these bacteria is one isolated by Schatz & Bovell (1952) and named by them *Hydrogenomonas facilis* because of the facility with which it may be cultured and experimented on. Most of the discussion in this chapter will be devoted to this organism since there is a reasonable amount of information about it and it is probably typical of the hydrogen bacteria.

Culture characteristics of H. facilis

H. facilis grows autotrophically, on mineral media solidified with silica gel, in an oxygen/hydrogen/carbon dioxide atmo-sphere ; it grows heterotrophically in air on ordinary nutrient media. Obligate aerobe, Gram-negative, short rod, motile by one or two polar flagellae ; cells occur singly or in short chains.

Heterotrophic growth is possible in an atmosphere of 100 per cent oxygen ; for autotrophic growth the concentration of

* I.e. ' Explosive-gas bacteria ', from the explosive properties of mixtures of hydrogen and oxygen.

oxygen in the atmosphere must not exceed 30 per cent, other-wise growth will cease. *H. facilis* has a great avidity for oxygen and will grow on the traces of oxygen found in commercial hydrogen or carbon dioxide. Culture plates of the organism may actually be used to deoxygenate desiccators required for anaerobic experiments.

Either ammonium salts or nitrate will serve as a nitrogen source for both autotrophic and heterotrophic growth. Under 10 per cent carbon dioxide plus 90 per cent air, there is no growth on a mineral medium fortified with ammonium salts, nitrite, or thiosulphate ; the organism has, therefore, none of the characteristics of the nitrifying bacteria (Chapter 5) or the thiobacilli (Chapter 3).

Wilson, Stout, Powelson & Koffler (1953) showed, by an elegant cross-plating technique, that *H. facilis* is a genuinely facultative autotroph and that cultures of it are not mixtures of autotrophs and heterotrophs.

The metabolic activities of H. facilis

Schatz & Bovell (1952) examined the metabolic activities of *H. facilis* by means of the Warburg technique. The organism proved to be an extraordinarily vigorous one ; in an atmosphere of hydrogen and oxygen, each cell consumed about one-sixteenth of its own dry weight of hydrogen per hour and about one-half of its own dry weight of oxygen. Under anaerobic conditions, with methylene blue as a hydrogen acceptor in place of oxygen, the hydrogen consumption increased to four times its aerobic value, i.e. each cell was consuming one-quarter of its own dry weight of hydrogen per hour. As an anaerobic hydrogen acceptor, methylene blue proved to be unique ; in a hydrogen atmosphere there was no significant gas uptake with acetate, pyruvate, fumarate, malate, oxaloacetate, α-ketoglutarate, formate, acetaldehyde, acetone, sulphate, nitrite, or sulphur. On the other hand, under aerobic conditions (i.e. in air), *H. facilis* oxidized acetate, lactate, pyruvate, succinate, malate, fumarate, oxaloacetate, and α-ketoglutarate, at roughly the same rate. Glucose was oxidized more slowly, but the rate increased as the experiment progressed. Oxalate and formate were oxidized very slowly, citrate, acetone, nitrite, thiosulphate,

and sulphur were not oxidized at all. These results suggest, first, that *H. facilis* cannot carry out the primary oxidation reactions of *Nitrobacter* (Chapter 5) or the thiobacilli (Chapter 3). Secondly, they suggest either that the cells are impermeable to some components of the Krebs tricarboxylic acid cycle, or that only fragments of this cycle are operative in the organisms. It is also clear that if hydrogen gas is used directly by *H. facilis* to generate reducing power in a transferable form (e.g. to generate DPNH or TPNH), this reducing power cannot *by itself* bring about a reversal of such reversible oxidation steps of the Krebs cycle as exist in the organism.

Cells held anaerobically under hydrogen reduced nitrate to nitrite but could not reduce the nitrite ; growing cells, however, must be able to do so because they can use nitrate as a sole source of nitrogen. Nitrite reduction must therefore be a ' growth bound ' process requiring the co-occurrence of some reaction that does not take place in resting cells. Nor were cells held anaerobically under hydrogen able to reduce carbon dioxide : traces of oxygen were essential for carbon dioxide reduction. No autotrophic growth took place in an atmosphere of 10 per cent carbon dioxide plus 90 per cent hydrogen, even when nitrate (which can act as a hydrogen acceptor) was present.

In Chapter 2 it was pointed out that the primary oxidation reaction of an autotroph might bring about carbon dioxide reduction in one of two ways. It might provide reducing power directly that could be transferred by carrier systems to the carbon-dioxide reducing systems. Alternatively, the substrate might be oxidized directly by oxygen and the energy released conserved as high-energy bonds ; these bonds could then be used to split water into an oxidizing component and a reducing component. The former would then be disposed of in some way and the latter would be used to effect the reduction of carbon dioxide. For the hydrogen bacteria, these schemes can be formulated :

Scheme A : Direct reduction
$$H_2 + A \text{ (hydrogen carrier)} = AH_2$$
$$CO_2 + 2AH_2 = (CH_2O) + H_2O + 2A$$
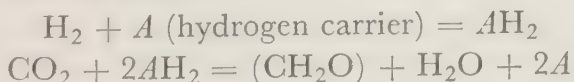
Here the first stage would be catalysed by hydrogenase, an enzyme found in a number of bacteria. It brings about the activation of molecular hydrogen, e.g.

$$\text{TPN}^+ + \text{H}_2 \xrightarrow[\text{system}]{\text{hydrogenase}} \text{TPNH} + \text{H}^+$$
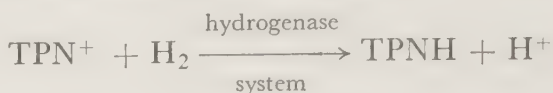
so that it may be transferred, as shown, to hydrogen-carrier systems. Its properties have been reviewed by Umbreit (1951).

Scheme B : Energy transfer

In this scheme it is supposed that hydrogen is first oxidized to water through some cytochrome system (an oxidation probably involving the initial activation of hydrogen by hydrogenase) with a release of chemical (probably phosphate) bond energy. About 35 kcal of bond energy would be released per atom of oxygen consumed if we assume that the energy released by the oxidation is much the same in *H. facilis* as it is in other organisms in which the generation of phosphate bond energy by cytochrome systems has been studied. The energy so generated is then supposed to be used to split water into a reducing component and an oxidizing component. The oxidizing component is disposed of and the reducing component used to effect CO_2 assimilation. If the reducing component is at an oxidation level near that of hydrogen, and the oxidizing component at an oxidation level near that of oxygen (say at rH of 5 and 35 respectively), about 40 kcal would be required for the splitting of each water molecule. The scheme may therefore be written :

    (i) $6H_2 + 3O_2$ —(cytochrome)$\longrightarrow 6H_2O + 210$ kcal

    (ii)   160 kcal (from (i))* $+ 4H_2O \longrightarrow 4[H] + 4[OH]$

    (iii) $CO_2 + 4[H] \longrightarrow (CH_2O) + H_2O$

    (iv) $4[OH]$ (from (ii)) $\longrightarrow 2H_2O + O_2$

Scheme B is a better explanation of the observed facts than is Scheme A, since Scheme B postulates some reason why the

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* In this scheme it is supposed that energy is transferred from reaction (i) to reaction (ii) with an efficiency of some 70 per cent. On the basis of results with other organisms, this figure seems reasonable.

assimilation of carbon dioxide by *H. facilis* demands the presence of oxygen. The sum of the reactions in Scheme A is

$$2H_2 + CO_2 = (CH_2O) + H_2O$$
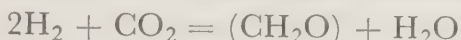
whereas the sum of the reactions in Scheme B is

$$6H_2O + 2O_2 + CO_2 = (CH_2O) + 5H_2O$$
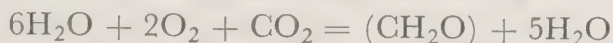
According to Scheme A, therefore, anaerobic growth is theoretically possible. According to Scheme B, growing cells of *H. facilis* would necessarily consume oxygen. They are known to do this, and, moreover, the 'sum' reaction of Scheme B is precisely the reaction quantitatively observed in the experiments of Schatz (1952). Schatz did not, however, propound any such theoretical scheme as that given above as an explanation of his experimental observations.

H. facilis will oxidize hydrogen and lactate simultaneously (Wilson *et al.* 1953) ; in this it resembles *H. flava* (Kluyver & Manten, 1942). Schatz, Isenberg & Trelawney (1953) allowed *H. facilis* to oxidize lactate in the presence of carbon dioxide containing isotopic carbon and were able to show that unusually large amounts of carbon dioxide were taken up by the organism during the oxidation, far larger than would be expected for ordinary 'heterotrophic fixation'. This is interesting because it suggests the *H. facilis* was behaving rather like the type of organism postulated on p. 14, i.e. using the energy released by an organic oxidation to bring about the 'autotrophic' assimilation of carbon dioxide.

Free-energy efficiency of the hydrogen bacteria

Baas-Becking & Parks (1927) calculated the overall free-energy efficiency of the hydrogen bacteria to be about 30 per cent—much higher than that of the other autotrophs. Schatz (1952), working with *H. facilis*, devised a most ingenious method for following the uptakes of oxygen, carbon dioxide, and hydrogen, all in the one Warburg vessel. His results also lead to the conclusion that the energy efficiency of the organisms is about 30 per cent. In the case of the hydrogen bacteria there is no doubt that the energy efficiency, short-term or overall, is high. This may be connected with the fact that the activities of the organisms do not produce any change in the

composition of the medium since the end-product of the primary oxidation is water (see p. 19).

Hydrogenase

The enzyme concerned in the activation of molecular hydrogen is, as has been mentioned, hydrogenase. In his review of this enzyme, Umbreit (1951) points out that of the various reactions shown in the following scheme, only reaction (1) can strictly be said to be catalysed by hydrogenase itself:

$$\overset{(1)}{H_2 \rightleftharpoons} \overset{(2)}{2H \rightleftharpoons} \overset{(3)}{2H^+ + 2e \rightleftharpoons} H \text{ carriers}$$

$$(4) \updownarrow$$
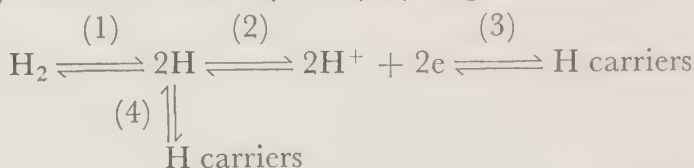
$$H \text{ carriers}$$

Whether reactions (2) and (3), or reaction (4), leading to the transfer of hydrogen to hydrogen carriers, were catalysed by the same enzyme as reaction (1) was doubtful when Umbreit wrote his review. It was thought that if one followed hydrogenase activity in impure* hydrogenase preparations by observing the rate of reduction of methylene blue, oxygen, or some easily reducible cellular component such as fumarate by molecular hydrogen, and tried to deduce the nature of hydrogenase itself from the effects of various poisons on the hydrogenase system, any inhibition observed under these circumstances might well be due to inhibition of carrier systems (present in the hydrogenase preparation) as to inhibition of the hydrogenase itself.

For this reason, Hobermann & Rittenberg (1943) tried to eliminate the possible effects of carriers by following the rate at which deuterium exchanged between the gas phase and water phase in resting suspensions of cells with hydrogenase activity. In this system, the following reactions occur:

$$H_2 \rightleftharpoons 2H^+ + 2e \qquad (a)$$
$$\updownarrow$$
$$2H_2O \rightleftharpoons 2H^+ + 2OH^- \qquad (b)$$
$$\updownarrow$$
$$D_2 \rightleftharpoons 2D^+ + 2e \qquad (c)$$
$$\updownarrow$$
$$2D_2O \rightleftharpoons 2D^+ + 2OD^- \qquad (d)$$
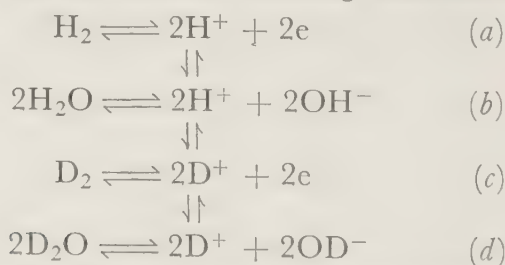
* Until very recently, only impure preparations of hydrogenase have been available.

Since reactions (*a*) and (*c*) are catalysed by hydrogenase, or, at least, are catalysed by hydrogenase *plus* the enzyme catalysing reaction (2) of the previous paragraph (if, indeed, this enzyme is actually different from hydrogenase), it is possible to follow the activity of hydrogenase by deuterium exchange without involving any hydrogen carrier systems.

By this technique, Hobermann & Rittenberg found that hydrogenase was not inhibited by 1 per cent sodium fluoride, 2 per cent urethane, $M/1000$ iodoacetate, $M/13$ malonate, or $M/100$ thiosulphate. It was inhibited by silver ions (100 per cent inhibition at $M/100$). When the hydrogenase-containing cells were shaken in air, their hydrogenase was temporarily inactivated, but it could be reactivated by mild reducing conditions. In its oxidized (temporarily inactivated) state, the hydrogenase could be permanently inactivated by cyanide; in its reduced (active) state the hydrogenase was insensitive to cyanide. This suggested that the hydrogenase system contained an iron porphyrin component, since cyanide is known to combine with iron porphyrin enzymes only when the iron is in the reduced state. Moreover, the hydrogenase activity was poisoned by carbon monoxide but this poisoning could be reversed by light ; such behaviour is again typical of what might be expected of an iron porphyrin enzyme.

This evidence, together with the observation of Waring & Werkman (1944) that iron-deficient micro-organisms tend to have a low hydrogenase activity, if it did not prove, seemed to indicate strongly that hydrogenase was an iron-containing enzyme. However, despite these strong indications, recent work suggests that hydrogenase itself may be a molybdenum enzyme rather than an iron enzyme. Shug, Wilson, Green & Mahler (1954) attempted to purify the hydrogenase of *Clostridium pasteurianum*. During the purification procedure adopted, molybdenum was gradually lost from the preparation, and, what is more important, so was the ability of the hydrogenase to reduce oxidized cytochrome in the presence of hydrogen. However, at all stages of the purification the hydrogenase retained its ability to reduce methylene blue in the presence of hydrogen. The ability to reduce oxidized cytochrome could be restored by the addition of molybdenum as

molybdenum trioxide. Boiled extracts of the hydrogenase showed the presence of flavin adenine dinucleotide.

It therefore seems probable that hydrogenase itself is a flavin adenine dinucleotide enzyme containing, as metallic component, molybdenum. The molybdenum is apparently necessary to make the enzyme work as a single-electron transfer system capable of reducing cytochrome. This may be summarized as

$$H_2 + \text{FAD-enz.} \longrightarrow \text{FAD-enz.} H_2$$
$$\text{FAD-enz.} H_2 + MB \longrightarrow \text{FAD-enz.} + MBH_2$$
$$H_2 + \text{FAD-Mo-enz.} \longrightarrow \text{FAD-Mo-enz.} H_2$$

$$\text{FAD-Mo-enz.} H_2 \begin{cases} + \text{cyt.Fe}^{+++} \\ + \text{cyt.Fe}^{+++} \end{cases}$$

$$\longrightarrow \text{FAD-Mo-enz.} \begin{cases} + \text{cyt.Fe}^{++} + H^+ \\ + \text{cyt.Fe}^{++} + H^+ \end{cases}$$
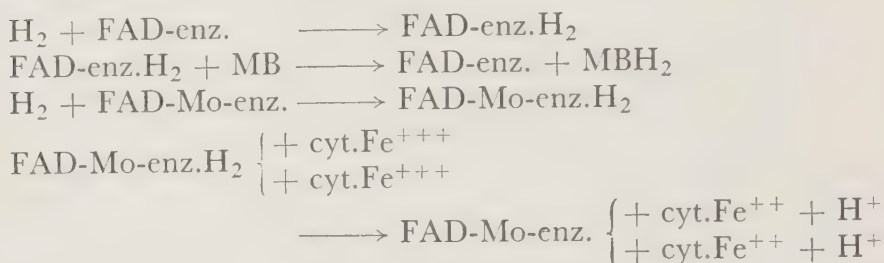
Where

FAD-enz.	= hydrogenase minus molybdenum
FAD-Mo-enz.	= hydrogenase incorporating molybdenum
MB	= methylene blue
MBH_2	= reduced (leuco) methylene blue
cyt.Fe^{+++}	= oxidized cytochrome
cyt.Fe^{++}	= reduced cytochrome

From this it follows that hydrogenase can generate hydrogen ions from hydrogen gas only by the intervention of cytochrome, and that the exchange reaction studied by Hobermann & Rittenberg (1943) was necessarily sensitive to poisons known to affect iron porphyrin enzyme systems since an iron porphyrin enzyme (cytochrome) was an essential part of the exchanging system.

Whether molybdenum is the active metal in all hydrogenases is not known. The work of Shug *et al.* indicates that in *Cl. pasteurianum*, at least, molybdenum is the metal concerned, but it does not follow, therefore, that in all organisms hydrogenase is a flavin adenine dinucleotide enzyme containing molybdenum. It seems probable, on the grounds of comparative biochemistry, that all hydrogenases are flavin adenine dinucleotide enzymes, but it may well be that in organisms other than *Cl. pasteurianum*, metals other than molybdenum are used to

transform a two-electron acceptance into a one-electron transfer. Variations of this kind are known. For instance, in many organisms, nitrate reductase is a molybdenum containing enzyme ; in other organisms it is a very similar enzyme but with iron replacing the molybdenum (Prof. F. Egami, private communication).

However, it is worth noting that the one hydrogenase that has been investigated at all intensively is capable of reducing oxidized cytochrome directly by a one-electron transfer. This harmonizes with the theory, already expressed, that the autotrophic hydrogen bacteria utilize the hydrogen to reduce oxidized cytochrome and obtain the energy for their autotrophic reduction of carbon dioxide from the chemical bond energy generated by the reoxidation, at the expense of molecular oxygen, of the reduced cytochrome so produced.

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THE NITRIFYING BACTERIA

THERE are two autotrophic bacteria, found in soil, sewage, dung, mud, and similar habitats, whose activities are almost invariably considered together. *Nitrosomonas*, which lives by oxidizing ammonia to nitrite, and *Nitrobacter*, which lives by oxidizing nitrite to nitrate. These are two quite distinct organisms; neither can oxidize the substrate of the other, but they are considered together because their combined activities bring about the well-known process of nitrification, i.e. the conversion of ammonia to nitrate, which occurs in most soils, in sewages, and in similar environments.

Both are small oval cells, *Nitrosomonas* ($1 \cdot 5 \times 1 \cdot 0\mu$) being a little larger than *Nitrobacter* ($1 \cdot 0 \times 0 \cdot 8\mu$). They are usually isolated on silica gel plates (Meiklejohn, 1950) or on agar plates (Jensen, 1950), and grown in liquid culture in thin layers or under forced aeration in bottles (Lees, 1952). Both are usually said to be Gram-negative, but the *Nitrosomonas* isolated by Jensen was weakly Gram-positive. Some species are motile.

Other similar but not identical organisms have been described from time to time. Whether these other organisms really exist, were slight variants, or were contaminants, is something of a vexed question (Bisset & Grace, 1954 ; Meiklejohn, 1954), but since almost all the biochemical studies have been carried out on *Nitrosomonas* and *Nitrobacter*, attention will be confined to these two.

Ammonia oxidation by Nitrosomonas

The primary oxidation step of *Nitrosomonas* is

$$NH_4^+ + 1\tfrac{1}{2}O_2 = 2H^+ + H_2O + NO_2^- + 66 \text{ kcal}$$
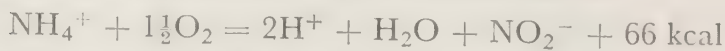
Since biological oxidations proceed by the removal of two hydrogen atoms or electrons at a time, the primary oxidation

step of *Nitrosomonas* cannot be a one-stage process. This has, of course, been realized for a long time ; nearly thirty years ago Kluyver & Donker (1926) proposed a theory to account for ammonia oxidation involving three steps :

(1) ammonia → hydroxylamine
(2) hydroxylamine → hyponitrite
(3) hyponitrite → nitrite

Let us now consider the evidence for the occurrence of these steps in ammonia oxidation by *Nitrosomonas*.

(1) *ammonia → hydroxylamine*

This reaction almost certainly occurs (Hofman & Lees, 1953). It is powerfully inhibited by thiourea or allylthiourea. *Nitrosomonas* cells suspended in M/100,000 thiourea or allyl-thiourea solutions would not oxidize any ammonia, yet under these conditions the oxidation of hydroxylamine to nitrite continued at an undiminished rate. (It should be noted that hydroxylamine oxidation by *Nitrosomonas* can be demonstrated only if the concentration of hydroxylamine is low. At concentrations *circa* M/10,000, hydroxylamine is oxidized as rapidly as ammonia ; at higher concentrations the hydroxylamine begins to poison the cells and the oxidation rate falls off.) The oxidation of hydroxylamine to nitrite is inhibited by hydrazine. In the presence of hydrazine no oxidation of hydroxylamine to nitrite occurs. Hydrazine is not, however, a powerful inhibitor of ammonia oxidation, so that, in the presence of hydrazine, cells metabolizing ammonia accumulate hydroxylamine. The first step in nitrite formation by *Nitrosomonas* may therefore be formulated :

ammonia ——|→ hydroxylamine ——|→ (nitrite)

Inhibited by   thiourea   hydrazine
                      allylthiourea
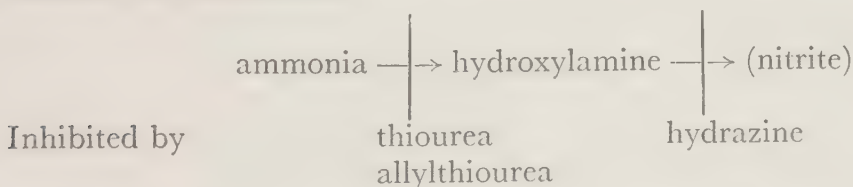
There are inferential grounds for believing that the system oxidizing ammonia to hydroxylamine involves a copper enzyme. The oxidation of ammonia in soil and in suspensions of *Nitrosomonas* is generally inhibited by chelating agents (Lees,

1946, 1952), especially so by the thioureas, which are known to have a peculiarly high affinity for copper. However, the enzyme system oxidizing ammonia has not been isolated and whether it actually does contain copper is therefore not known.

The loss of free energy involved in the oxidation of ammonia to hydroxylamine is small (Martin, Buehrer & Caster, 1942). The free-energy efficiency of *Nitrosomonas* would therefore scarcely be affected if this stage of the oxidation of ammonia were not coupled to any hydrogen carrier systems or to any systems generating bond energy. If it is assumed that there is no such coupling, the oxidation of ammonia to hydroxylamine in *Nitrosomonas* may be formulated :

$$NH_4^+ + \tfrac{1}{2}O_2 = NH_2OH + H^+$$

This formulation is attractive for several reasons. First, the ionic species are correct because within the pH range of maximum *Nitrosomonas* activity, the bulk of the ammonia (pK = 9·5) will be cationic and the bulk of the hydroxylamine (pK = 5·9) will be uncharged. Secondly, the enzyme catalysing the reaction is depicted as dealing directly with molecular oxygen ; many copper enzymes (monophenol oxidase, polyphenol oxidase, tyrosinase, ascorbic acid oxidase) are known to do this. Thirdly, the results of Meyerhof (1917) suggest that although NH_4^+ cannot easily penetrate the cell wall of *Nitrosomonas*, the uncharged NH_3 molecule can and does. Engel (1941) believed that ammonia oxidation in *Nitrosomonas* took place on the cell surface and not within the cytoplasm. In view of these findings, the direct oxidation of ammonia, as formulated above, may be looked on as a device whereby the nitrogenous substrate of the primary oxidation is changed from a cationic form, scarcely capable of penetrating the cell wall, to a neutral form (NH_2OH) capable of penetrating the cell wall with much the same ease as NH_3. Oxidation of the NH_2OH, which involves almost all the free-energy loss of ammonia oxidation, could then take place in the cell cytoplasm where suitable energy coupling mechanisms would be readily available.

The interconversion of ammonia and hydroxylamine is not confined to *Nitrosomonas*. There is a hydroxylamine reductase

system in *Bacillus pumilis* that reduces hydroxylamine to ammonia and will use methylene blue as a hydrogen donor for the process (Taniguchi, Mitsui, Toyoda Yamada & Egami, 1953)*. Klausmeier & Bard (1954) obtained an ' ammonium dehydrogenase ' system from *B. subtilis* ; this system catalysed the reversible reaction

$$NH_4OH + DPN^+ = NH_2OH + DPNH + H^+$$

Whether either of these systems bears any resemblance to the ' ammonia oxidase ' of *Nitrosomonas* is not known because attempts to prepare the ' ammonia oxidase ' in a cell-free state have so far been unsuccessful.

(2) *hydroxylamine → hyponitrite*

This reaction should really be written

$$\text{Hydroxylamine} \to X$$

where X may be hyponitrite ($H_2N_2O_2$), dihydroxyammonia ($NH(OH)_2$), or nitroxyl (NOH), since any of these compounds could, in theory, be the end-product of the reaction. In fact, the identity of X is not known, but there is some theoretical interest in considering which of the three possibilities is the most likely one.

(*a*) *Hydroxylamine → dihydroxyammonia.*—This may be formulated as a dehydrogenation of a hydrated hydroxylamine molecule :

$$NH_2.OH.H_2O = NH(OH)_2 + 2[H]$$
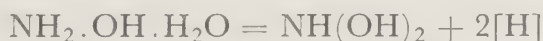
Such a reaction may well occur ; the difficulty of proving that it does is due to the fact that dihydroxyammonia is so unstable that it has never been prepared. Conceivably it might have a transient existence on an enzyme surface prior to dehydrogenation to nitrite,

$$NH(OH)_2 = HNO_2 + 2[H]$$

but such existence could only be inferred from other data. Free dihydroxyammonia could never be isolated from, or added to, the metabolizing systems of *Nitrosomonas*.

* According to a personal communication from Professor Egami, this system is not poisoned by allylthiourea.

(b) *Hydroxylamine → nitroxyl.*—Nitroxyl could result from the direct dehydrogenation of hydroxylamine :

$$NH_2.OH = NOH + 2[H]$$

Nitrite formation from nitroxyl would involve either hydration followed by dehydrogenation, or a direct coupling between nitroxyl and molecular oxygen :

$$NOH.H_2O = HNO_2 + 2[H]$$

or

$$NOH + \tfrac{1}{2}O_2 = HNO_2$$
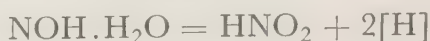
The difficulty of proving that nitroxyl lies in the reaction sequence hydroxylamine → nitrite is precisely that met with in the case of dihydroxyammonia ; nitroxyl cannot be prepared because it is too unstable. It is interesting to note, however, that Kluyver & Verhoeven (1954) postulate nitroxyl as the intermediate between nitrite and hydroxylamine in the reduction of nitrate to ammonia by *Micrococcus denitrificans*.

(c) *Hydroxylamine → hyponitrite.*—Largely because hyponitrite is a relatively stable compound, the possibility that hydroxylamine is converted to hyponitrite is the one that has received the most experimental attention. Since biological oxidations proceed by the removal of two hydrogen atoms or electrons, this reaction, if it occurs, must be a two-stage process involving the intermediate formation of nitroxyl or dihydroxyhydrazine :

$$NH_2.OH = NOH + 2[H] \qquad 2NOH = HON:NOH$$
$$\text{(hyponitrous acid)}$$

or

$$2NH_2.OH = (HO)NH.NH(OH) + 2[H]$$
$$(HO)NH.NH(OH) = HON:NOH + 2[H]$$
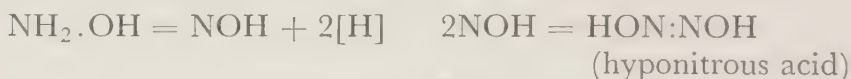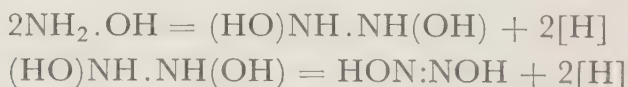
The question is, however, does hyponitrite occur at all as an intermediate in ammonia oxidation by *Nitrosomonas* ? Mumford (1914) and Corbet (1934, 1935) both seem to have found hyponitrite in nitrifying cultures. On the other hand, the evidence collected by Mellor (1928, p. 289) suggests that hyponitrite can be formed by purely chemical reactions in solutions containing small concentrations of both hydroxylamine and nitrite, especially if chalk is also present. These

conditions are precisely the conditions obtaining in growing cultures of *Nitrosomonas*, so it is quite possible that the hyponitrite observed by Mumford and Corbet was formed by chemical rather than biological processes. Moreover, there are no reports that *Nitrosomonas* is capable of forming nitrite from hyponitrite, and it is the author's experience that when hyponitrite is added to washed supensions of *Nitrosomonas* no nitrite formation occurs. Dr H. L. Jensen (personal communication) has been equally unsuccessful in parallel experiments. The explanation may lie in an inability of hyponitrite to penetrate the cell wall of *Nitrosomonas*, but it seems rather more probable that hyponitrite is not, in fact, an intermediate in the oxidation of ammonia to nitrite by *Nitrosomonas*, just as it is not an intermediate in the reduction of nitrite to ammonia in *Pseudomonas stutzeri* (Allen & van Niel, 1952), *Bacillus pumilis* (Taniguchi *et al.*, 1953), or *Micrococcus denitrificans* (Kluyver & Verhoeven, 1954).

There is one final possibility not so far considered. The steps postulated between hydroxylamine and nitrite have all been formulated as removals of two hydrogen atoms. This is the classical representation and it applies to all biological oxidations and reductions that take place outside the terminal oxidation systems, i.e. the metal flavoprotein + cytochrome systems that finally transfer the electrons to molecular oxygen. Within these systems, however, electrons are transferred one at a time. Now it is not difficult to imagine that a molecule as small as hydroxylamine might be taken up directly, as a metal-hydroxylamine complex, by such systems; indeed, hydroxylamine is known to be taken up by the iron porphyrin enzymes such as catalase. The oxidation of the hydroxylamine might then proceed as successive losses of a single electron from the original hydroxylamine-metal complex, each loss being followed by the uptake of a hydroxyl ion. If this were the mechanism of hydroxylamine oxidation in *Nitrosomonas*, the formulation of 'intermediates' in the process would be transformed into a formulation of electron distribution within the complex between the metal and the nitrogenous moiety; indeed, no independently existing 'intermediates' would occur.

Nitrite oxidation by Nitrobacter

The primary oxidation step of *Nitrobacter* is

$$NO_2^- + \tfrac{1}{2}O_2 = NO_3^- + 17 \text{ kcal}$$
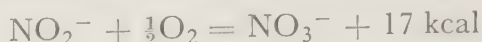
This may be formulated either as the dehydrogenation of a hydrated nitrite ion :

$$H_2O \cdot NO_2^- = NO_3^- + 2[H]$$
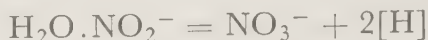
or, as was suggested at the end of the previous section, two one-electron transfers mediated by metal enzymes :

$$NO_2^- = NO_2 + e$$
$$NO_2 + H_2O = NO_3^- + 2H^+ + e$$
$$\tfrac{1}{2}O_2 + 2H^+ + 2e = H_2O$$
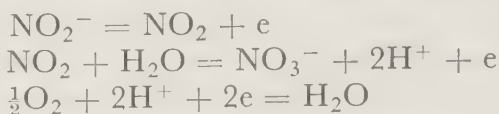
The second formulation has the advantage that it is in line with the mechanism of nitrate reduction. Nitrate reduction to nitrite is carried out by a number of micro-organisms, and the enzyme responsible, nitrate reductase, has been isolated. It proves to be a molybdenum flavoprotein that catalyses the transfer of electrons (from TPNH) to nitrate which accepts them one at a time ; its action is thus precisely the reverse of that suggested for nitrite oxidation.

Until recently no work whatever had been done on the ' nitrite oxidase ' of *Nitrobacter* and therefore the information about it is scanty. It now appears (Lees & Simpson, 1955) that nitrite oxidation is coupled in some way to the cytochrome system of the cells. Washed cells suspended in water show three absorption bands at 589, 551, and 520–5 mμ, the band at 551 mμ, corresponding in position to the α-band of cytochrome c, is rather faint. If nitrite is added, this band immediately becomes intense, indicating that the cytochrome has been changed from the oxidized to the reduced state. Normally the rate of oxidation of the nitrite is more or less constant from the beginning of the oxidation to the end, but if chlorate is present the rate gradually diminishes as the experiment proceeds. If the concentrations of nitrite and chlorate are suitably chosen, the oxidation can be brought to a stop long before it is complete. Cells whose oxidation of nitrite has been stopped in this way by chlorate show no absorption band at 551 mμ in the

presence of nitrite, nor can the band be restored even by treatment of the cell suspension with dithionite (the standard chemical reducing agent for cytochromes). The explanation appears to be that chlorate can partly replace oxygen as a final hydrogen acceptor and so become reduced to chlorite ; the chlorite then destroys the cytochrome. The fact that cells whose cytochrome system has been damaged in this way can no longer oxidize nitrite is further evidence that nitrite oxidation is coupled with the cytochrome system.

This action of chlorate on nitrite *oxidation* by resting washed suspensions of *Nitrobacter* is apparently different from the action of chlorate on proliferating cells. Soil percolation experiments (Lees & Quastel, 1945) showed that, at concentrations of M/100,000, chlorate acted as a bacteriostatic agent to the growth of *Nitrobacter* in soils without affecting nitrite oxidation by *Nitrobacter* ; that is, M/100,000 chlorate could markedly delay the rate at which the *Nitrobacter* population of a soil increased (in response to percolation of the soil with nitrite solution) but that it had no effect on the rate at which an already established population oxidized percolated nitrite. This bacteriostatic effect of chlorate was antagonized by nitrate, so that if nitrite, nitrate and chlorate, in suitable concentrations, were percolated through a soil with an initially low *Nitrobacter* population, the population would build up at the same rate as in a parallel experiment where the nitrite alone was percolated, although in a soil percolated with nitrite and chlorate (nitrate omitted) the build-up would be delayed. These results have since been confirmed on freshly inoculated cultures of *Nitrobacter* in mineral media ; low concentrations of chlorate increase the length of the ' lag-phase ' of such cultures, and nitrate abolishes the effect. This effect is not understood. It may be that *Nitrobacter* uses nitrate rather than nitrite as a source of nutritional nitrogen and that chlorate somehow blocks the utilization of nitrate. But, whatever the explanation, the action of chlorate here is not the same as its action on nitrite oxidation. To demonstrate its action on oxidation, chlorate must be used at concentrations of at least M/500 and preferably at M/100, i.e. between 100 and 1000 times more concentrated than for a demonstration of its bacteriostatic effect. Moreover,
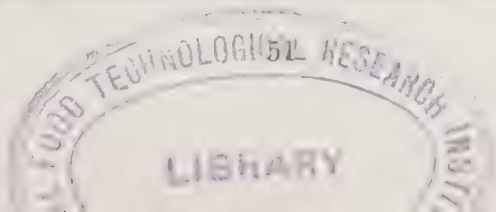
the effect of chlorate on nitrite oxidation is not affected at all by nitrate.

Culture requirements of the nitrifying bacteria

(a) *Optimum* pH.—Although the optimum pH of both *Nitrosomonas* and *Nitrobacter* may vary from strain to strain (Winogradsky & Winogradsky, 1933) both organisms are usually grown in faintly alkaline media. The pH optima of 8·0 for *Nitrosomonas* and 7·7 for *Nitrobacter*, based on the results of Warburg experiments (Hofman & Lees, 1953 ; Lees, 1954), seem to agree very well with the results obtained by Martin *et al.* (1942) in their investigations on nitrite formation and oxidation in alkaline soils. Both organisms are fairly tolerant of conditions more acid than their optima, there being appreciable activity at pH 6·5, but both are equally intolerant of too great alkalinity ; at pH 9·5 there is little activity.

(b) *Surface.*—Both *Nitrosomonas* and *Nitrobacter* tend to adhere to solid surfaces ; indeed, as far as *Nitrosomonas* is concerned, the provision of a surface for it on which to grow seems to be almost a necessity, although *Nitrobacter* is less exacting in this respect. For this reason they are usually grown in dilute carbonate/phosphate media (adjusted to the appropriate pH) to which is added a little solid calcium carbonate. The organisms then grow on the surface of the calcium carbonate particles. *Nitrobacter* may be removed from the particles merely by agitating the calcium carbonate suspension for a few minutes in a household homogenizer ; *Nitrosomonas* adheres far more strongly. It is therefore not surprising that, in soil, the oxidation of ammonia is confined almost entirely to that taking place at the surface of the soil (Lees & Quastel, 1946) ; oxidation in the soil solution is almost nil.

(c) *Trace elements.*—Calcium is necessary for *Nitrosomonas* (Kingma Boltjes, 1935), and Bömeke (1949) has shown that magnesium, iron, and phosphate are all essential for both *Nitrosomonas* and *Nitrobacter*. Meiklejohn (1953) estimates the optimal concentration of iron at 6 ppm in the medium for both organisms ; she also found that at 560 ppm iron became inhibitory. This may have been a genuine inhibition by the iron itself ; alternatively, one should remember that in

aerated alkaline solutions almost all the iron added will be precipitated as hydroxide, so that the addition of 560 ppm of iron as ferrous salt is equivalent to adding sufficient mineral acid to make the medium M/50 in that acid. On the other hand, much of the induced acidity will be neutralized by the calcium carbonate precipitate in the medium, so the resultant pH change may be quite small. It would be most interesting to have Dr Meiklejohn's experiments repeated in media at (say) pH 6 where both organisms will grow to some extent and where reasonable ionic concentrations of iron are possible. This does not necessarily complete the list of trace elements that the nitrifying organisms require, and others may well be essential, although no-one has yet shown them to be so. If, for instance, ammonia oxidation by *Nitrosomonas* is mediated by a copper enzyme, copper must be an essential element for *Nitrosomonas*. If the nitrite oxidase of *Nitrobacter* resembles the nitrate reductase of other organisms, molybdenum will be essential for *Nitrobacter*. A thorough investigation of the trace element requirements of the nitrifying organisms is most desirable ; an accurate knowledge of these requirements could indicate the type of enzymes to be sought in the primary oxidation systems.

(d) *Growth factors.*—Both *Nitrosomonas* and *Nitrobacter* can develop in a purely mineral medium. Whether under these conditions they achieve maximal growth rates and whether they can continue to develop indefinitely under these conditions are questions that cannot be answered unequivocally. Fred & Davenport (1921), Murray (1923), Kingma Boltjes (1935), and Hes (1937) have all shown that various organic compounds enhance the growth of the nitrifying organisms. These results agree with the common observation that nitrification occurs most readily in soils, dung-heaps, sewage, and mixed (rather than pure) cultures where coexistent organisms could supply growth factors inadequately synthesized by the nitrifiers themselves. There seems to be little doubt that some organic compound or compounds can supplement the internal biochemical economy of the nitrifiers and so induce an augmented growth rate. Whether the nitrifiers have an absolute requirement for such growth factors is uncertain, but there is no doubt that pure cultures of the nitrifiers do tend to die out. Despite

53

the dozens of reports of isolation of one or other of the nitrifiers, there is (to the author's knowledge) only one pure culture of *Nitrosomonas* in existence at the time of writing ; and, to the author's knowledge, no pure culture of *Nitrobacter* exists at all.

Substances inhibitory to the nitrifying bacteria

A compound may inhibit *Nitrosomonas* or *Nitrobacter* in one of two ways. It may inhibit the primary oxidation reaction or it may inhibit the general metabolism of the cell. Both classes of compound will affect the growth of the organisms, but a member of the second class will not necessarily show any effect on the primary oxidation reaction as studied in short-term experiments with resting cells.

The first really comprehensive studies on inhibitors of the primary oxidation reactions of both bacteria were carried out by Meyerhof (1916, 1917) by means of the Warburg technique. His results covered a large range of substances and are too lengthy even to summarize adequately. In general, however, he concluded that lipid soluble substances, including the more common narcotics, were poisonous to both organisms, rather more so to *Nitrosomonas* than to *Nitrobacter*. Presumably these substances acted by disturbing the metabolism of the cell as a whole and so influencing the primary oxidation reaction, rather than by specifically interfering with the primary oxidation reaction. Meyerhof also showed that glucose, glycerol, mannitol, acetate, butyrate, and valerate were virtually without effect on either organism. This was an important discovery, because a paper by Winogradsky & Omeliansky (1899) had purported to show that simple substances such as glucose, asparagine, and butyrate had markedly deleterious effects on nitrification in culture ; this paper had given rise to the widespread (and manifestly absurd) belief that ' organic materials poison the nitrifying bacteria '. The belief is absurd because nitrification takes place in dung-heaps, yet it has become so deep rooted that it is still sometimes repeated in modern books on bacteriology. Chelating agents (including the amino acid histidine) inhibit the primary oxidation reaction of *Nitrosomonas* (Lees, 1952), and cyanate inhibits the primary oxidation reaction of *Nitrobacter* (Lees & Simpson, 1955). The action of

cyanate is different from the chlorate action already mentioned as it does not increase as the experiment progresses and it can always be reversed by washing the cells with water.

Peptone is generally agreed to inhibit the growth of the organisms in culture, and Kingma Boltjes (1935) showed that the inhibition was related to the free amino acid content of the peptone used. He also found that *Nitrosomonas* would grow in mineral media to which 4 per cent glucose had been added, despite statements of Winogradsky & Omeliansky (1899) to the contrary. Jensen (1950) found that various amino acids inhibited the growth of *Nitrosomonas* and later reported a particularly high toxicity for cysteine, which appeared to have two concentration maxima of toxic effect. One of these maxima was thought to be attributable to cysteine, the other appeared to be due to breakdown products of the cysteine rather than to the cysteine *per se* (Jensen & Sörensen, 1952). Jensen (1950) also found that although glucose sterilized by filtration did not inhibit the growth of *Nitrosomonas*, even at 5 per cent concentration, glucose sterilized by autoclaving was much more toxic. This he tentatively ascribed to the partial conversion of the glucose during autoclaving to mannose—a sugar he found to be surprisingly toxic to the growth of *Nitrosomonas* (completely inhibitory at M/80).

There are many more reports in the literature of various inhibitors (usually of the growth) of the nitrifying organisms, but until more is known of the internal metabolism of the cells, the effects reported are likely to remain incomprehensible.

Composition of the nitrifying bacteria

Hofman (1953) has analysed hydrolysates of *Nitrosomonas* cells for amino acids and carbohydrates. The amino acid spectrum proved to be normal, all the common amino acids were present, no unusual amino acid was detected. Galactose, ribose, rhamnose, and xylose were identified but, curiously enough, glucose was not found. Desoxyribose was presumably present, but as only acid hydrolysis was used, which destroys desoxyribose, this sugar did not appear in the analysis.

No similar analysis has been carried out on *Nitrobacter*, but, as already stated, the organism contains a cytochrome system.

The free-energy efficiencies of the nitrifying bacteria

The end-product of *Nitrosomonas* primary oxidation reaction
—nitrite—is generally thought of as a rather toxic substance ;
it is also small and easily diffusible. It might be expected,
therefore, that as the growth of a *Nitrosomonas* culture proceeded,
and nitrite accumulated in the medium, a low nitrite concen-
tration within the cell cytoplasm could be maintained only by
an increasing expenditure of energy on some mechanism that
excreted nitrite. It could therefore be expected that the free-
energy efficiency of *Nitrosomonas* would fall as the nitrite con-
centration of the culture rose, because energy made available
by the primary oxidation reaction would be increasingly
diverted from carbon dioxide assimilating mechanisms to
nitrite-excreting mechanisms. Whether this explanation is
correct or not, the fact remains that the overall energy efficiency
of young cultures of *Nitrosomonas* is higher than that of old
cultures (Hofman & Lees, 1952) ; moreover, the rather con-
flicting determinations of overall free-energy efficiency for
Nitrosomonas made by other workers (Baas-Becking & Parks,
1927 ; Nelson, 1931 ; Hes, 1937 ; Bömeke, 1951) could all
be reconciled with each other and with the new determinations
of Hofman & Lees if due account were taken of the nitrite
concentration in the culture when the determination was made.
In the very early stages of growth, the overall free-energy
efficiency may be at least 20 per cent; in older cultures it drops
to the usually quoted figure of about 7 per cent.

No short-term efficiency figure for *Nitrobacter* is available.
Baas-Becking and Parks (1927) put the overall efficiency at
6 per cent.

Internal metabolism of the nitrifying bacteria

Bömeke (1939) found, as one might expect, that thick suspen-
sions of cells prepared by centrifuging old cultures of the
nitrifiers showed small but measurable respirations in the
absence of ammonia (*Nitrosomonas* cells) and nitrite (*Nitrobacter*
cells). This was the ' rest respiration ' that all cells, being open
systems, must show. He then tried to increase this respiration
by adding various organic substances to the suspensions. As
modern methods of increasing the accessibility of internal

enzyme systems, such as freeze drying and low-temperature homogenization, were not available at that time, the results were not very encouraging. There were increases in respiration when certain substances were added, notably lactate and acetate, but the increases were very small. Nevertheless, this was pioneer work that deserves repetition by modern techniques.

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OTHER CHEMOSYNTHETIC BACTERIA

THE bacteria described in the previous chapters are the best known and best characterized of the chemosynthetic bacteria. There are, however, others that are certainly, and more that are possibly, capable of following the same mode of life.

The anaerobic sulphate bacterium *Desulphovibrio desulphuricans* reduces sulphate to sulphide while oxidizing organic compounds. Butlin, Adams & Thomas (1949) showed that it could live autotrophically on the energy released when it reduced sulphate to sulphide at the expense of hydrogen :

$$4H_2 + H_2SO_4 \rightarrow H_2S + 4H_2O + 60 \text{ kcal}$$

and Sisler & ZoBell (1950, 1951) showed that similar organisms had the further remarkable property of ' fixing ' molecular nitrogen during this reduction, i.e. of reducing molecular nitrogen to a form in which it was available for nutrition of the organism. These organisms are therefore capable of the extraordinary feat of growing in a carbonate-sulphate medium under an atmosphere of hydrogen and nitrogen.

Recent work by Verhoeven, Koster & van Niefelt (1954) has suggested that *Micrococcus denitrificans* may be able to live autotrophically by oxidizing hydrogen at the expense of either oxygen or nitrate. An organism capable of oxidizing either hydrogen or carbon monoxide has been isolated by Kistner (1953) ; this recalls the work of Kaserer (1906), who discovered that *Bacillus oligocarbophilus* was capable of growing autotrophically on the energy released by the oxidation of carbon monoxide to carbon dioxide :

$$CO + \tfrac{1}{2}O_2 \rightarrow CO_2 + 74 \text{ kcal}$$

It is possible that further research may show the existence of autotrophic bacteria capable of utilizing even more extraordinary inorganic transformations as sole energy sources for their life, growth, and proliferation.

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THE PHOTOSYNTHETIC BACTERIA

PHOTOSYNTHETIC bacteria were first described by Engelmann in 1883. He noted that their pigmentation had a well-defined absorption spectrum and showed that the organisms, when placed on a microscope slide illuminated by a spectrum, collected in those regions of the spectrum where they most strongly absorbed light. This suggested that they were similar to green plants and effected photosynthesis in the same way. The overall equation for photosynthesis in green plants is

$$H_2O + CO_2 \xrightarrow[\text{energy}]{\text{light}} (CH_2O) + O_2$$
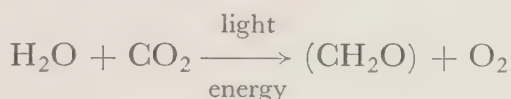
i.e. photosynthesis that proceeds with the evolution of free oxygen. Engelmann believed he had demonstrated the evolution of oxygen by his coloured bacteria, but later workers were not able to confirm this and the whole subject fell into a state of confusion that was not cleared up until the now classical studies of van Niel (1931, 1935, 1936) brought order out of chaos.

Van Niel found he could isolate from mud two types of photosynthetic bacteria, one purple, one green. Four conditions were necessary for their isolation and growth : (a) illumination, (b) a mineral medium containing carbonate, phosphate, chloride, and sulphide, (c) a slightly alkaline reaction, (d) strict anaerobiosis. He showed that oxygen was not evolved during growth, but that the sulphide of the medium was oxidized instead. In the green bacteria, the sulphide was oxidized to sulphur and the overall equation for carbon dioxide assimilation and sulphide oxidation was

$$2H_2S + CO_2 \xrightarrow[\text{energy}]{\text{light}} (CH_2O) + H_2O + 2S$$
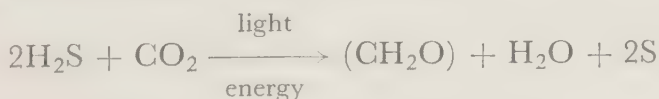
whereas in the purple bacteria the oxidation of the sulphide proceeded right through to sulphate :

$$H_2S + 2H_2O + 2CO_2 \xrightarrow[\text{energy}]{\text{light}} 2(CH_2O) + H_2SO_4$$
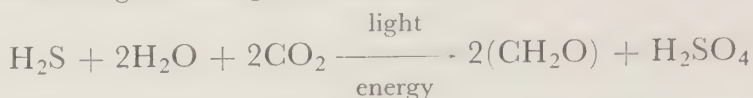
That is, in the photosynthetic bacteria, an inorganic oxidation replaced the oxygen evolution of the green plants.

More recent investigations have shown that the metabolisms of the green and purple sulphur bacteria (as they are usually called) are not so sharply differentiated as these classifications suggest. Moreover, compounds other than hydrogen sulphide will serve as oxidizable substrates, but it is convenient to deal with the two groups separately ; the green bacteria will be first considered.

The green sulphur bacteria

Bergey's Manual (6th Edition) accords the green sulphur bacteria the status of a family, the Chlorobacteriaceae, comprising six genera. Whether this classification is justified is somewhat doubtful (Larsen, 1954 ; Bisset & Grace, 1954), and in any case only one genus, *Chlorobium*, has been studied in any detail. Most of our knowledge of *Chlorobium* is due to Helge Larsen, who, working in van Niel's laboratory, has produced the first really coherent account of the green sulphur bacteria (Larsen, 1953).

Morphology of Chlorobium

Two species were recognized by Larsen, *C. limicola* and *C. thiosulphatophilum*. This species differentiation is biochemical since it is based on the type of sulphur compound the organism is able to use in its photosynthesis. Morphologically, the species are indistinguishable ; both are non-motile, each is a rod- or oval-shaped cell with dimensions $0 \cdot 7\mu \times 0 \cdot 9$ to $1 \cdot 5\mu$.

Isolation of Chlorobium *species*

A summary of Larsen's isolations, which were based on a technique originally devised by Winogradsky, is included here because it gives some idea of the difficulties encountered in working with these organisms.

Pure cellulose (filter paper), calcium sulphate, and mud were placed in a tall glass cylinder which was then filled with water and illuminated ; the following sequence of events then took place. First aerobic micro-organisms soon removed any traces of oxygen, and the contents of the cylinder thus became anaerobic. Anaerobic organisms then attacked the cellulose with the evolution of carbon dioxide and hydrogen. A further group of anaerobes reduced the sulphate of the calcium sulphate to sulphide by means of the hydrogen, and finally the photo-synthetic sulphur bacteria, which grew on the walls of the cylinder where illumination was maximal, assimilated carbon dioxide while oxidizing the sulphide to sulphur or sulphate. Colonies of purple sulphur bacteria always appeared on the walls of the cylinder, colonies of green bacteria appeared only spasmodically. Those that did occur were picked off with a fine pipette and seeded into a suitable sulphide-containing liquid medium and kept under anaerobic conditions in the light ; in this medium the green sulphur bacteria developed. They were further purified by transfer to sulphide-agar covered with paraffin wax to ensure anaerobiosis. Two types of colony developed in the agar : a fast-growing type which, after further transfers to sulphide agar, gave pure cultures of *C. limicola*, and a slower-growing type which, on similar purification, gave pure cultures of *C. thiosulphatophilum*, so called because it could use thiosulphate (which thus became oxidized to sulphate) as electron donor in its photosynthetic processes. *C. limicola* could use only sulphide as electron donor.

Nutrient requirements of Chlorobium *species*

The organisms could be grown on a completely mineral medium (ammonium chloride, magnesium chloride, sodium bicarbonate, potassium phosphate) supplemented with other inorganic compounds. Sodium chloride (1 per cent in the medium) was necessary to ensure maximal growth. Iron (50 μg/litre) was necessary to bring about the formation of the ' Chlorobium chlorophyll ' that gives the organisms their green colour and is presumably the primary photosynthetic pigment.*

* Iron is apparently necessary for the operation of enzyme systems concerned in chlorophyll synthesis. Chlorophyll itself is, of course, a magnesium porphyrin compound.

63

Calcium was also required. Other trace elements added were boron, zinc, cobalt, copper, and manganese. The electron donor (sodium thiosulphate or sodium sulphide) was added at 0·1 per cent ; concentrations of sodium thiosulphate higher than 0·4 per cent were toxic to *C. thiosulphatophilum*, in contrast to the high concentrations of thiosulphate (up to 3 per cent) tolerated by thiobacilli. The pH of the medium was adjusted to about 7·5. The medium was prepared and placed in sealed bottles the day before it was required, a trace of sulphide being added where thiosulphate was the electron donor. This pre-preparation was necessary so that traces of oxygen in the water could be oxidized away by the sulphide present before the medium was inoculated. The organisms are strict anaerobes and the smallest amount of oxygen will prevent growth. After the medium had been inoculated with organism, it was, of course, constantly illuminated.

Metabolism of Chlorobium *species*

(a) *Sulphide as electron donor.*—*C. thiosulphatophilum* will always oxidize sulphide to sulphate. As sulphur transiently appears in cultures of this organism growing with sulphide as electron donor, it seems probable that the reactions involved are

$$(1) \quad CO_2 + 2H_2S \xrightarrow[\text{energy}]{\text{light}} (CH_2O) + 2S + H_2O$$

$$(2) \quad 3CO_2 + 2S + 5H_2O \xrightarrow[\text{energy}]{\text{light}} 3(CH_2O) + 2H_2SO_4$$
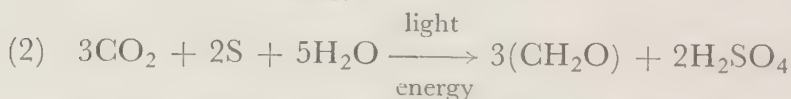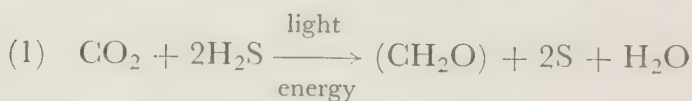
Warburg experiments, conducted on suspensions of the organism prepared by centrifuging cells from cultures, were carried out in an atmosphere of 95 per cent nitrogen plus 5 per cent carbon dioxide. Sulphide was used as electron donor. Equation 1 results in a decrease in acidity and should lead to an increase in the amount of carbon dioxide ' bound ' as carbonates by the medium ; equation 2 results in an increase in acidity and should lead to a decrease in the amount of bound carbon dioxide. The results of the Warburg experiments agreed with these predictions. In virtually unbuffered suspensions, illuminated and supplied with sulphide, there was

first an increase and then a decrease in 'bound' carbon dioxide. Parallel experiments with suspensions buffered by phosphate showed merely a gradual decrease in bound carbon dioxide as the pH slowly fell. Determinations of the carbon dioxide actually *assimilated* during sulphide oxidation showed that the assimilation rate diminished when all the sulphide had been converted to sulphur and the production of sulphuric acid began. The total carbon assimilated in these Warburg experiments was only 60 per cent of what should have been assimilated according to the amount of sulphide oxidized. It seems that the cells were metabolically damaged during centrifuging and resuspension. Analyses of growing cultures showed a close parallelism between carbon assimilation and sulphide oxidation in accordance with the theoretical equation.

C. limicola was not so thoroughly examined. Experiments on growing cultures suggested, however, that while the general metabolism of sulphide was much as in *C. thiosulphatophilum*, the conversion of sulphur to sulphate was rarely, if ever, complete. *C. limicola* tends, therefore, to accumulate sulphur in the medium and thus more nearly accords with the original concept of a green sulphur bacterium.

The mechanism of sulphur oxidation is obscure. The first stage, the oxidation of sulphide to sulphur, obviously occurs in both organisms. *C. thiosulphatophilum* oxidizes both thiosulphate and tetrathionate, so presumably the path of sulphur oxidation to sulphate is through these two compounds. *C. limicola* is, however, quite unable to metabolize either compound. This indicates either that these two compounds do not occur in the metabolic pathway here followed by the sulphur, or that the cell wall is impermeable to them.

(*b*) *Thiosulphate as electron donor.*—Analyses of growing cultures of *C. thiosulphatophilum* showed that the conversions taking place were adequately described by

$$2CO_2 + Na_2S_2O_3 + 3H_2O \xrightarrow[\text{energy}]{\text{light}} 2(CH_2O) + Na_2SO_4 + H_2SO_4$$
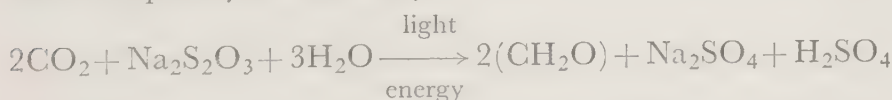
Warburg experiments with buffered and unbuffered suspensions similar to those described in the last section suggested that during the oxidation of thiosulphate to sulphur there was no

production of alkali. This suggests that there is no appreciable accumulation of polythionates during the oxidation because the formation of a divalent polythionate ion from the divalent thiosulphate ion involves the release of a certain number of sodium ions with a consequent increase in alkalinity. Moreover, trithionate, dithionate, and sulphite all failed to act as electron donors for *C. thiosulphatophilum*. Tetrathionate, however, functioned not only as an electron donor in Warburg experiments but also functioned as an electron donor for the growth of cultures. As in the case of the thiobacilli, the path of oxidation of sulphur compounds remains very obscure.

(*c*) *Hydrogen as an electron donor.*—Both organisms can carry out photosynthesis with hydrogen as an electron donor. Here the overall equation is

$$2H_2 + CO_2 \xrightarrow[\text{energy}]{\text{light}} (CH_2O) + H_2O$$
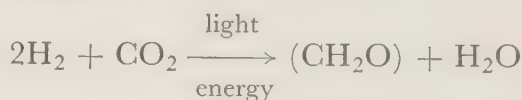
The ability to utilize hydrogen often goes hand in hand with the ability to fix molecular nitrogen, i.e. to use it as a source of nutritional nitrogen. Lindstrom, Burris & Wilson (1950) have shown this ability in a Chlorobacterium that was, in fact, *C. thiosulphatophilum*.

Organic compounds and C. thiosulphatophilum

Purple sulphur bacteria are able to use organic compounds as electron donors in their photosynthesis. Tests were made to find out whether *C. thiosulphatophilum* had any similar ability. Various carbohydrates were tested, all with negative results. Indeed, the carbohydrates proved to be toxic, as no growth took place in the presence of the carbohydrates even when thiosulphate was added as electron donor. Various organic acids were then tried, and in this case there was just possibly a little growth with acetic, propionic, and lactic acids as electron donors. The curious fact emerged, however, that although propionic acid could not act as an adequate electron donor for photosynthetic activity, it was nevertheless carboxylated by *C. thiosulphatophilum*, principally to succinic acid :

$$CH_3.CH_2.COOH + CO_2 = HOOC.CH_2.CH_2.COOH$$

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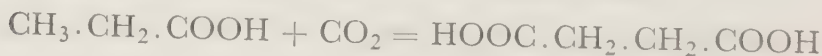
This carboxylation apparently lies outside the normal metabolic pathway of carbon dioxide assimilation, because in the presence of thiosulphate as electron donor, the assimilation of carbon dioxide (linked with the oxidation of thiosulphate) and the carboxylation of propionic acid to succinic proceeded independently of each other.

Similar experiments were not carried out on *C. limicola*.

Endogenous metabolism of C. thiosulphatophilum

In the light, and in the absence of electron donors, the metabolism of the organism is low, a very small output of carbon dioxide being the only detectable activity. In the dark, similarly in the absence of electron donors, there is an appreciable output of carbon dioxide and hydrogen sulphide. The source of the latter is probably various intermediates (in oxidative pathways leading to sulphate) temporarily stored within the cell. The fact that the output of hydrogen sulphide in the dark is slightly augmented by thiosulphate is in agreement with this suggestion. *C. thiosulphatophilum* apparently possesses, therefore, a ' reversible ' metabolism, as do green plants. Green plants in the light photosynthesize according to the equation

$$CO_2 + H_2O = (CH_2O) + O_2$$

and in the dark respire according to the equation

$$(CH_2O) + O_2 = CO_2 + H_2O$$
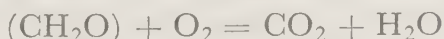
C. thiosulphatophilum photosynthesizes according to the equation

$$CO_2 + \text{reduced S compounds}$$
$$= (CH_2O) + \text{oxidized S compounds}$$
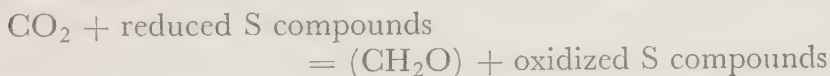
and respires in the dark according to the equation

$$(CH_2O) + \text{oxidized S compounds}$$
$$= CO_2 + \text{reduced S compounds (including } H_2S)$$
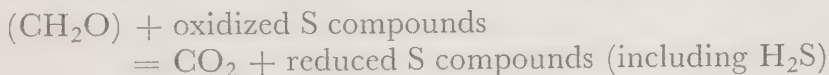
Similar experiments on *C. limicola* were not carried out.

The purple sulphur bacteria (*Thiorhodaceae*)

These organisms, the Thiorhodaceae, are morphologically far more diverse than the green sulphur bacteria. They are more easily isolated and a considerable amount of work has

been done on them. An adequate list of references to these investigations is outside the scope of this book ; guiding references will be found in the review of the autotrophic bacteria by van Niel (1954).

Their metabolism may be summarized by saying that they possess all the photosynthetic abilities and some of the dark-metabolism characteristics of *C. thiosulphatophilum* plus the ability to use a variety of organic compounds as electron donors in place of inorganic sulpur compounds. In other words, if we represent a suitable organic compound as H_2A, the purple sulphur bacteria can carry out a photosynthesis summarized by

$$2H_2A + CO_2 \xrightarrow[\text{energy}]{\text{light}} (CH_2O) + H_2O + 2A$$
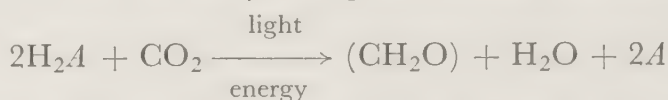
There are hints of this sort of metabolism in the behaviour of *C. thiosulphatophilum*; in the purple sulphur bacteria the metabolism is fully developed.

This ability to photosynthesize with an organic compound as electron donor leads to a curious result. In the formulation given above, the dehydrogenated substrate A may well be further dehydrogenated and so provide reducing power for the assimilation of more carbon dioxide by photosynthesis. This process may continue until A finally represents simply carbon dioxide. Now if H_2A is initially at the same oxidation level as the final cellular material synthesized, the total photo-synthetic process will be summarized by

$$C'H_2O + C''O_2 \xrightarrow[\text{energy}]{\text{light}} (C''H_2O) + C'O_2$$
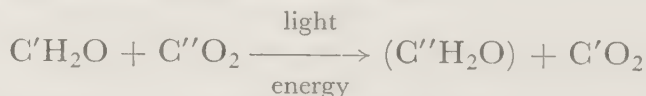
where $C'H_2O$ represents H_2A of the previous equation, i.e. the original organic carbon will be turned into carbon dioxide and the original carbon dioxide turned into cell material with no net output or uptake of carbon dioxide. This is an idealized representation ; it may well be that some of the organic fragments arising from the dehydrogenation of the $C'H_2O$ may themselves enter the cell and be reduced at the expense of further oxidation of $C'H_2O$. But the net result as far as carbon dioxide uptake or output is concerned will be the same. Similarly, if the organic electron donor is more oxidized than

the cell material eventually formed, there will be a net carbon dioxide output during photosynthesis ; if it is less oxidized, there will be a net uptake. With the simple organic compounds that have been tested as electron donors, this prediction is fulfilled. If lactate (roughly the same level of oxidation as the total cell material) is used, photosynthesis proceeds with little change in the total amount of carbon dioxide present ; with succinate (more oxidized than the cell material) there is an output of carbon dioxide ; with butyrate (less oxidized than the cell material) there is an uptake.

Here then is a metabolism apparently bordering on the heterotrophic ; a simple organic compound ($\pm$ carbon dioxide) is being transformed into cell material. Yet it is a strictly photosynthetic process ; light is essential and the process proceeds until all the simple organic compound has disappeared. It can be accurately formulated as a reduction of carbon dioxide by the reducing power liberated on the oxidation of the simple organic electron donor *to* carbon dioxide.

The non-sulphur purple bacteria (*Athiorhodaceae*)

The metabolism of these organisms is even nearer to the heterotrophic way of life. All use a variety of organic compounds as electron donors for photosynthesis, all (with the single known exception of *Rhodomicrobium vannielii* described by Duchow & Douglas, 1949) require various B vitamins as growth factors ; some utilize sulphide or thiosulphate as electron donors in place of organic compounds, some utilize hydrogen as electron donor (as will the green and purple sulphur bacteria). It is thus clear that, biochemically, certain Athiorhodaceae are very difficult to distinguish from the Thiorhodaceae, and morphologically similar difficulties arise (Bisset & Grace, 1954). Where certain of the Athiorhodaceae differ markedly from the Thiorhodaceae is, however, in their ability to grow *aerobically* in the dark on the same substrates that they oxidize *anaerobically* (i.e. utilize as electron donors in their photosynthesis) in the light. It is interesting to note, perhaps, that *R. vannielii*, which atypically requires no growth factor and is also morphologically atypical, does not possess this ability. Biochemically, indeed, *R. vannielii* is much more

nearly related to the purple sulphur bacteria than it is to the non-sulphur purple bacteria.

Of all the photosynthetic bacteria, this group has been the most thoroughly investigated from a biochemical standpoint. Yet we shall here do little more than record its existence and characteristics. In so far as the Athiorhodaceae can be classed as 'autotrophic' micro-organisms, their metabolism is similar to that of the purple sulphur bacteria ; but their known requirement for growth factors places them even a little nearer than the purple sulphur bacteria to the heterotrophic way of life. Their aerobic dark metabolism, when they are living by the oxidation of an organic compound (and, incidentally, showing the same requirement for growth factors as they do in the light), is entirely heterotrophic. It is interesting to note, however, that the biochemical pathways by which the Athiorhodaceae assimilate the carbon of an organic compound are probably different in the light than in the dark. Some aspects of the photometabolism (and dark metabolism) of the Athiorhodaceae have been discussed in an interesting article by Elsden (1954).

Photosynthetic mechanisms in the photosynthetic bacteria

The pigments of the photosynthetic bacteria are concentrated in 'grana' of about 0.05μ diameter ; each cell of *Rhodospirillum rubrum* contains about 5,000 such grana. These grana, which do not appear when the cells are grown in the dark, correspond to the chloroplasts of higher plants and, like the chloroplasts, contain both chlorophylls and carotenoids as protein conjugates. The chlorophyll of the purple bacteria (sulphur and non-sulphur) is probably always the same 'bacteriochlorophyll', whereas the 'Chlorobium chlorophyll' or 'bacterioviridin' of the green sulphur bacteria is different. The carotenoids of the photosynthetic bacteria have not yet been thoroughly investigated, the only well-characterized one (from the purple bacteria) being spirilloxanthin. Nothing is known about the carotenoids of the green sulphur bacteria. It should perhaps be mentioned that the chlorophyll of all the photosynthetic bacteria, like that of plants, is green. The colour of the purple bacteria is due to the green's being

masked by accessory pigments such as carotenoids and phyco-
bilins. These pigments have been discussed by Blinks (1954).
The generally accepted plan of photosynthesis is that light
energy absorbed by chlorophyll, or absorbed by carotenoids
and passed on to chlorophyll by some mechanism, is used to
split water into an oxidized component and a reduced com-
ponent; the former is disposed of in some way, the latter is used
in the reduction of carbon dioxide. In green plants, according
to Calvin and his school, thioctic acid (otherwise known as
α-lipoic acid, see review by Long, 1953) may play an important
role here; it is suggested that the energy obtained from the
light-activated chlorophyll is used to split the bridge between
the two sulphur atoms of the thioctic acid and that a water
molecule adds (as H and OH) on to the two freed and active
sulphur atoms:

$$R . \underset{S}{\overset{S}{\bigg\langle}} \bigg| - \left(\begin{array}{l} \text{energy from} \\ \text{chlorophyll} \end{array} \right) \to R . \overset{S \cdot}{\underset{S \cdot}{\bigg\langle}} - (+ \, H_2O) \to R . \overset{S . H}{\underset{S . OH}{\bigg\langle}}$$
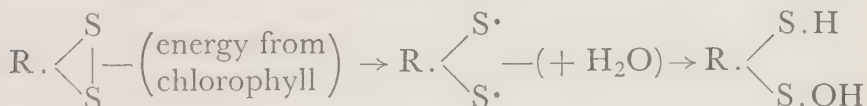
The —S.H then acts as a source of reducing power and the
—S.OH is (in green plants) eventually used as a source of the
oxygen liberated in photosynthesis; the original thioctic acid
is, of course, thus regenerated by the removal of the H and the
OH. A favourable effect of thioctic acid on photosynthesis has
indeed been noted recently by Bradley & Calvin (1954).
Whether bacterial photosynthesis involves thioctic acid is
not known, but excluding such details and following the plan
of van Niel (1949) *all* photosynthesis may be formulated as

$$H_2O - \left(\begin{array}{l} \text{light energy,} \\ \text{pigments, enzymes} \end{array} \right) \overset{\nearrow \, [H] \, \to \, \text{(electron acceptors reducing } CO_2)}{\searrow \, [OH] \, \to \, \text{(electron donors)}}$$
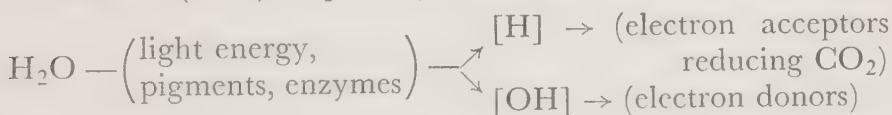
In green-plant photosynthesis the [OH] needs no electron
donor, it is merely transformed into molecular oxygen. In
bacterial photosynthesis the organisms lack the ability to
effect this transformation and are constrained to dispose of the
[OH] by using it to oxidize some substrate such as hydrogen
sulphide, sulphur, hydrogen, or (in the case of the purple
bacteria) an organic compound. There are hints (*see* Elsden,
1954) that the disposal of the [OH] may involve cytochrome.

71

This scheme postulates that the oxidation of the electron donor is remote from the reduction of carbon dioxide, and it is quite possible to imagine that the energy freed by the oxidation of the electron donor by the [OH] would be wasted. In fact, this proves to be the case. Wassink, Katz & Dorrestein (1942), working with purple sulphur bacteria, and Larsen (1953), working with green sulphur bacteria, have shown that nine quanta of light are always the minimum number necessary for the assimilation of one molecule of carbon dioxide no matter what the electron donor may be. The energy released by the oxidation of hydrogen to water is high, and as a consequence the reaction

$$CO_2 + 2H_2 \rightarrow (CH_2O) + H_2O$$

which summarizes bacterial photosynthesis with hydrogen as the electron donor, requires (in theory) only 2 kcal energy. The oxidation of thiosulphate to tetrathionate yields little energy, so that photosynthesis summarized as

$$CO_2 + 4S_2O_3^{--} + 3H_2O \rightarrow (CH_2O) + 2S_4O_6^{--} + 4OH^-$$
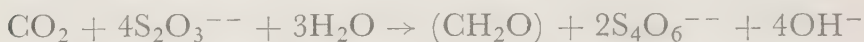
theoretically requires 100 kcal. In fact, nine quanta of light are required to effect either reaction ; it follows that the energy released by the oxidation of the electron donor is quite unavailable to the organism, a possibility precisely envisaged in van Niel's formulation.

The mechanism of carbon dioxide assimilation in green-plant photosynthesis is known to be broadly represented by the Calvin cycle (see Appendix). The mechanism in bacterial photosynthesis has not been investigated with anything approaching the same vigour, but such results as have been obtained do suggest that a similar scheme operates in this type of photosynthesis as well.

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THE EVOLUTION AND RELATIONS OF THE AUTOTROPHS

This chapter is largely speculative ; it is simply an attempt to bring some order into the apparently chaotic biochemistry of the organisms we have been considering. We shall try to answer two questions : Is it possible to imagine all autotrophs as being derived from one primeval autotrophic archetype ? If so, what biochemical patterns should we expect the autotrophs now to retain in common ?

Before attempting to answer these questions we must be quite clear that we are *not* here concerned with the Origin of Life. We are concerned with the evolution of present forms of life from past forms, these past forms being as remote from us in time as we can imagine cellular organisms to have existed on Earth. Those interested in the philosophical implications of the Origin of Life should consult (as a beginning) Penguin *New Biology* No. 16.

No particular originality is claimed for the views put forward in this chapter. They are based on the ideas, expressed or implied, in the papers of many workers. Prominent among these are van Niel, Umbreit, Kamen, Vernon, Elsden, and Calvin. This chapter is an attempt to weld all these ideas into a solid structure ; if, on close examination, the welds should prove to be riddled with blow-holes, the fault is probably my own.

Primitive organisms

At the moment there seems to be reasonable agreement that the primeval atmosphere of the Earth, although it contained carbon dioxide, was probably devoid, or almost devoid, of oxygen ; the oxygen now found in the atmosphere is thought to have been produced by the photosynthetic activities of organisms that liberate oxygen during photosynthesis (i.e. the green plants). This theory is in part based on the allegation that the amount of carbon originally assimilated by photosynthesis and now fossilized as coal and oil deposits corresponds,

within a factor of one or two, with the amount of oxygen in the atmosphere today. In other words, the equation

$$CO_2 + H_2O \text{ —(photosynthesis)} \rightarrow (CH_2O) + O_2$$

$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \qquad\quad \downarrow$

$\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ coal & oil atmosphere
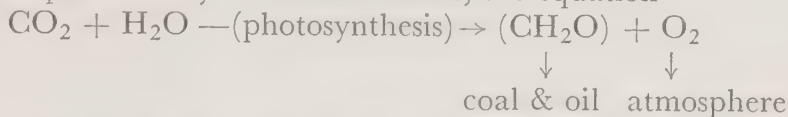
roughly balances on a world scale. It also seems probable that, in the early stages of the Earth's history, there was little organic material present and that, as a consequence, primitive organisms would be compelled to rely on non-organic sources of energy for their growth. In the absence of oxygen, the only source of energy likely to be adequate for any considerable synthesis of cell material would be sunlight ; we are thus driven to assume that the first organisms were photosynthetic organisms living in an anaerobic environment except in so far as their photosynthetic activities may have produced localized concentrations of oxygen near the organisms.

Photosynthetic activity

Let us assume, to begin with, that these primitive organisms had a photolytic mechanism for splitting water into an oxidized and reduced component much as present-day photosynthetic organisms have, and that their anabolic processes may be summarized as

Light energy $\qquad$ H_2O

$\qquad\qquad \searrow \qquad \nearrow$

$\qquad\left(\begin{array}{l}\text{chlorophyll,}\\ \text{carotenoids}\end{array}\right)$

[OH] $\qquad\qquad\qquad\qquad\qquad$ [H]

$\qquad\qquad\qquad\qquad\qquad CO_2 \searrow \mid \nearrow \sim X^*$

$\qquad\qquad\qquad\qquad\qquad$ small organic
$\qquad\qquad\qquad\qquad\qquad$ compounds

$\qquad\qquad\qquad\qquad\qquad \mid \nearrow \sim X$

$\qquad\qquad\qquad\qquad\qquad$ structural material
$\qquad\qquad\qquad\qquad\qquad$ of the cell (proteins,
$\qquad\qquad\qquad\qquad\qquad$ polysaccharides, fats, etc.)
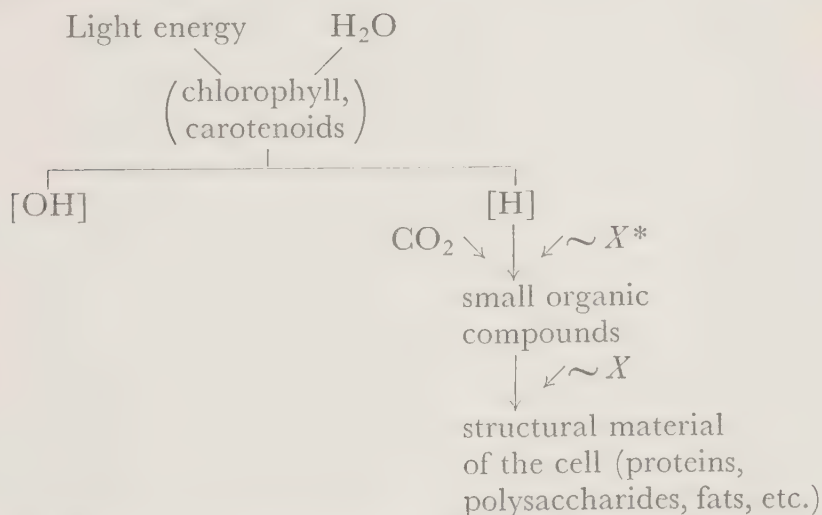
* $\sim X$ will be used to denote chemical bond energy, e.g. phosphate or sulphur bond energy.

Two problems arise from this formulation : (i) What is the source of $\sim X$? (ii) How is the [OH] moiety resulting from the photolytic split of the water to be disposed of ? Question (i) may be answered by supposing that, if [H] is transferred to DPN^+ or TPN^+ to yield DPNH or TPNH, $\sim X$ could be generated by the reoxidation of the DPNH or TPNH, the ultimate hydrogen acceptor in this oxidation being [OH]. The overall process here, i.e. the photolysis of water followed by a recombination of the photolytic products resulting in the generation of $\sim X$, is, of course, equivalent to a conversion of light energy into chemical energy.

Let us now turn to question (ii). Since any assimilation of carbon dioxide will consume [H], assimilation will always result in a surfeit of [OH]. One way of disposing of this surfeit would be *via* hydrogen peroxide ; Calvin and his school have suggested that hydrogen peroxide may be formed from the $-S.OH$ of the hydrated thioctic acid (*see* p. 71). Now, as is known, two enzymes, catalase and peroxidase, are capable of dealing with hydrogen peroxide. The former breaks it down to water and oxygen, the latter reduces it to water at the expense of some substrate. If we call the substrate AH_2, we can imagine the disposal of [OH] being accomplished by either of the following mechanisms :

$$2[OH] \rightarrow H_2O_2 \text{------- (catalase)} \longrightarrow H_2O + O_2$$

$$AH_2 \longrightarrow \text{(peroxidase)} \longrightarrow 2H_2O + A$$
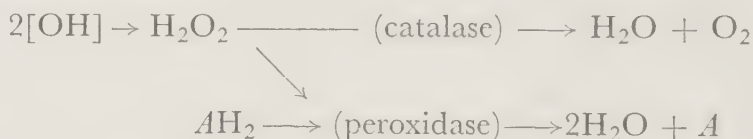
Both catalase and peroxidase are iron porphyrin enzymes and one might imagine that in primitive organisms there was one enzyme having properties of both ; it is in fact known that, when coupled with hydrogen-peroxide-producing systems such as xanthine oxidase, catalase can show the properties of a peroxidase. The term ' peroxidase ' is indeed used here to cover not only ' normal ' peroxidase, but all enzyme systems that might have an overall action similar to that of peroxidase. It would cover, for instance, the activities of a cytochrome system (another iron porphyrin enzyme) in which the oxidizing agent was not molecular oxygen but the [OH] moiety derived from the photolysis of water :

$$2\text{cyt}^{++} \quad + 2[\text{OH}] = 2\text{cyt}^{+++} \quad + 2\text{OH}^-$$
$$2\text{cyt}^{+++} + A\text{H}_2 \quad = 2\text{cyt}^{++} \quad + 2\text{H}^+ + A$$
$$\text{Sum } A\text{H}_2 + 2[\text{OH}] = A + 2\text{H}^+ + 2\text{OH}^-$$
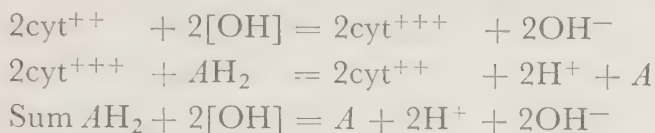
The differences between this cytochrome system and a normal aerobic cytochrome system are : (i) in all probability a different cytochrome oxidase, capable of being oxidized by [OH], would be involved, and (ii) as [OH] is at a lower oxidation level than molecular oxygen, it might well be that generation of chemical bond energy during reoxidation of the reduced cytochrome would be small or zero. Low or zero levels of energy generation would also result if the cytochrome had a high oxidation potential as has the ' cytochrome f ' of plants. A cytochrome similar to ' cytochrome f ' has been detected in photosynthetic bacteria by Kamen & Vernon (1954) and Vernon & Kamen (1954). The overall effect of a cytochrome system such as that postulated would thus be similar to that of a peroxidase, with the exception that [OH], which we may regard as ' potential ' hydrogen peroxide, rather than actual hydrogen peroxide would be used to oxidize $A\text{H}_2$.

We may imagine the primitive photosynthetic organisms as possessing both ' catalase ' and ' peroxidase ' mechanisms for disposing of surplus [OH]. If peroxidase activity were then lost by an organism, we should have an organism whose photosynthesis would correspond to that of the modern green plant ; surplus [OH] would be obligatorily disposed of by a ' catalase ' mechanism involving evolution of molecular oxygen. On the other hand, if catalase activity were lost, some electron donor $(A\text{H}_2)$ would be essential for the disposal of surplus [OH]. In the green sulphur bacteria, this may be either hydrogen or an inorganic sulphur compound ; in the purple sulphur bacteria it may, in addition, be a simple organic compound.

The purple non-sulphur bacteria, in the light and under anaerobic conditions, behave similarly to the purple sulphur bacteria. In the dark, however, they can live aerobically on the same substrates they oxidize anaerobically and photosynthetically in the light. We may therefore imagine that, in the dark, these substrates are oxidized by a ' normal ' cytochrome system generating $\sim X$, rather than by a ' peroxidase '

77

type of system such as operates in the light. It is interesting to note here that Kamen & Vernon (1954) and Vernon & Kamen (1954) have detected in photosynthetic bacteria two types of cytochrome oxidase, a photo-oxidase (active only in the light) and a 'thermal' or 'dark' oxidase. If we imagine the cytochrome system operating through the photo-oxidase and using [OH] as the final electron acceptor as equivalent to a 'peroxidase' system, and the cytochrome system operating through the thermal oxidase and using oxygen as the final electron acceptor as equivalent to a 'normal' cytochrome system, the activities of the purple non-sulphur bacteria are explained. The green sulphur bacteria and purple sulphur bacteria would, of course, be supposed to have no effective thermal oxidase ; this possibility is suggested by Kamen & Vernon.

The $\sim X$ generated by the operation of the 'normal' (dark) cytochrome system in the purple non-sulphur bacteria could then be used to split water into [H] and [OH] by some chemolytic system. This split would necessarily be stepwise since the lysis of water into an [H] component and an [OH] component both reasonably positioned on the rH scale would involve the expenditure of some 40 kcal/molecule of water (p. 38) and a chemical bond (phosphate or sulphur) cannot generally supply more than about 15 kcal. Photolysis can, of course, proceed in one step since a mole photon of red light can supply the whole 40 kcal in one operation.

In summary we may therefore say that photosynthetic bacteria metabolizing in the light would be expected to obtain all their energy from the light, since the electron donor, AH_2, is used merely to dispose of [OH] by a 'peroxidase' type of system in which all the energy resulting from the oxidation of AH_2 is wasted. In the dark, the purple non-sulphur bacteria may be supposed to be capable of tapping the energy released by the oxidation of AH_2 since in the dark the oxidation of AH_2 is *via* a 'normal' cytochrome system capable of generating $\sim X$.

In the chemosynthetic bacteria, we may imagine that because of evolutionary losses of enzymic abilities, only the metabolism typified by the dark metabolism of the purple non-sulphur bacteria remains. Furthermore, only inorganic, and not

organic, substrates will serve as electron donors. On the other hand, there are hints (p. 39) that *Hydrogenomonas facilis* may be able to use lactate as an electron donor in an otherwise ' auto-trophic ' metabolism. It is also interesting to note that the nitrifiers are alleged to be inhibited by light (*see* Meiklejohn, 1954, for a discussion of this point) ; it may be that traces of a
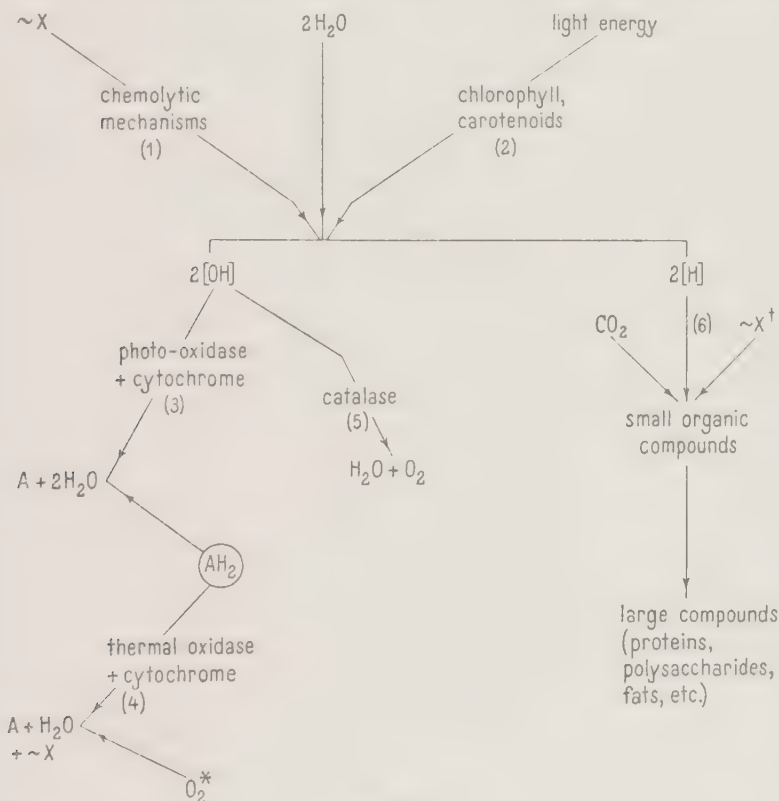
Figure 2

photometabolism remain in these organisms, or rather that traces of a light-activated ' peroxidase ' system remain whose operation inhibits the generation of $\sim X$ from the substrate being oxidized.

Figure 2 summarizes all the speculations of this chapter.

* Or SO_4^{--} in *Desulphovibrio desulphuricans* (this organism, although a strict anaerobe, possesses a cytochrome system : Postgate, 1954). In *Micrococcus deni-trificans*, O_2 can probably be replaced by NO_3^-.

† Generated by the recombination of [H] and [OH] in photosynthetic organisms.

Figure 2 may be taken to represent all the potential activities of a primeval autotroph. It is worth noting that reactions 2–5 are all mediated by metal porphyrin enzymes in modern organisms. It may be that, in the primeval autotroph, one inefficient metal porphyrin enzyme mediated all these reactions and that the modern enzymes have been derived from the one original enzyme by a process of specialization. On the other hand, it may be that specialization of these enzyme systems was already accomplished in the primeval autotrophs. This is a matter for conjecture.

The really interesting point about *Figure 2* is, however, that it covers the activities of all living organisms. All we have to do is assume that, in any particular type, one or more of the six mechanisms shown in the figure may be absent (their absence may be accounted for either by failure to specialize certain systems or by evolutionary loss of already specialized systems ; *see* previous paragraph). The abilities and inabilities of modern organisms are summarized in *Table I*.

Table I

| *Type of organism* | Reaction (+ = present, − = absent) | | | | | | AH$_2$ (I=*inorganic*, O=*organic*) |
	1	2	3	4	5	6 *	
Purple non-sulphur bacteria	+	+	+	+	?	+	I & O
Purple sulphur bacteria	−	+	+	−	−	+	I & O
Green sulphur bacteria	−	+	+	−	−	+	I
Green plants	−	+	+	−	+	+	*Nil*
Chemosynthetic bacteria	+	−	?	+	+	+	I
All heterotrophs	−	−	−	+	+	+	O

* If the cell is unable to synthesize *all* cellular material *via* reaction 6, such compounds as cannot be synthesized must be supplied pre-formed as, for example, ' essential amino acids ' or ' growth factors '.

There are, as we have seen, hints in various experimental findings that *Figure 2*, in some measure at least, truthfully corresponds with the facts. How truthfully it represents the facts can be decided only by further experiments. For the time being it may be regarded as a reasonable working hypothesis that has the merit of making autotrophic activity tolerably comprehensible.

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9

THE AUTOTROPHIC BACTERIA IN NATURE

THE biochemistry of the autotrophic bacteria has now been dealt with in about as much detail as available experimental results warrant. Strictly speaking, that is the limit of scope implied by the title of this book ; yet it seems not unreasonable, in conclusion, to devote a little space to some consideration of the parts played by these organisms in nature. Do they appreciably influence, by the chemical changes they bring about, other organisms living in association with them ? Or are they mere biochemical curiosities that can safely be written off as unimportant by the practically minded ? To these questions one may reply straight away that the autotrophic bacteria are much more than mere biochemical curiosities. On the contrary, the inorganic transformations they bring about may be of considerable quantitative importance in the overall biochemical changes taking place in the environment. As far as mankind is concerned, these transformations may be beneficial (the production of elemental sulphur by the photosynthetic bacteria) or undesirable (the corroding of concrete and stonework by thiobacilli) ; few are economically negligible. In this chapter some of the large-scale changes brought about by autotrophic bacteria working in their natural environments will be considered.

Typically, chemosynthetic bacteria are to be found in soil, mud, and sewage, where there are supplies of such inorganic compounds as they require for their primary energy-yielding reactions. Photosynthetic bacteria, having an absolute demand for light, are generally confined to illuminated translucent habitats such as streams and lakes, although some organisms will often be washed on to the surrounding mud, where they may continue to live for a time, even if they cannot grow.

The activities of the autotrophic bacteria will be considered, first, as they operate in soil, and secondly as they operate in lakes and streams.

Autotrophic bacterial activity in soil

Before autotrophic activity in soil can be properly understood, some knowledge of soil structure is necessary, as the structure of a soil inevitably influences the activities of organisms inhabiting the soil. Of course, it is impossible here to discuss the intricacies of soil structure, which is quite a separate subject. All that can be done is to indicate the type of structure that fertile agricultural and garden soils—in which autotrophic activity is generally most apparent and important—usually have.

Such soils consist of mineral particles cemented together by organic materials, these organic materials being subsumed under the heading of 'soil organic matter' or 'humus'. The mineral particles range from 'sand' (particles with diameters between $2 \cdot 0$ and $0 \cdot 2$ mm), through 'silt' to 'clay' (below $0 \cdot 002$ mm). The cementing of these particles by soil humus results in the formation of 'soil crumbs', i.e. spongy masses (having diameters from about $0 \cdot 5$ to $5 \cdot 0$ mm) of mixed mineral and organic materials. The description of 'spongy' as applied to these soil crumbs is tolerably exact, and each crumb is permeated with countless holes of various diameters very similar to those found in a sponge. The soil as a whole (or rather, the upper foot or so) consists of a mass of these soil crumbs, with the result that a cross-section of the soil appears rather similar to the idealized diagram of *Figure 3*, where the limits of each 'crumb' are delineated by a thickened line.

This system of soil crumbs has various properties, two of which are relevant to our present purpose. The first of these is simply that the system consists of solid materials with spaces between them. These spaces can be grouped under the heading of 'pore space' of the soil and subdivided into 'pore spaces' of various diameters. The spaces between the soil crumbs are clearly of relatively large diameters, of the order of millimetres or large fractions of a millimetre. From such

spaces water can freely drain under the influence of gravity alone, and, in a reasonably well drained soil, these spaces would be filled with air. The same applies to the larger holes permeating the soil crumbs, but, even in a freely drained soil, water is more and more likely to be held in the increasingly smaller holes in the crumbs by capillary attraction. Even in
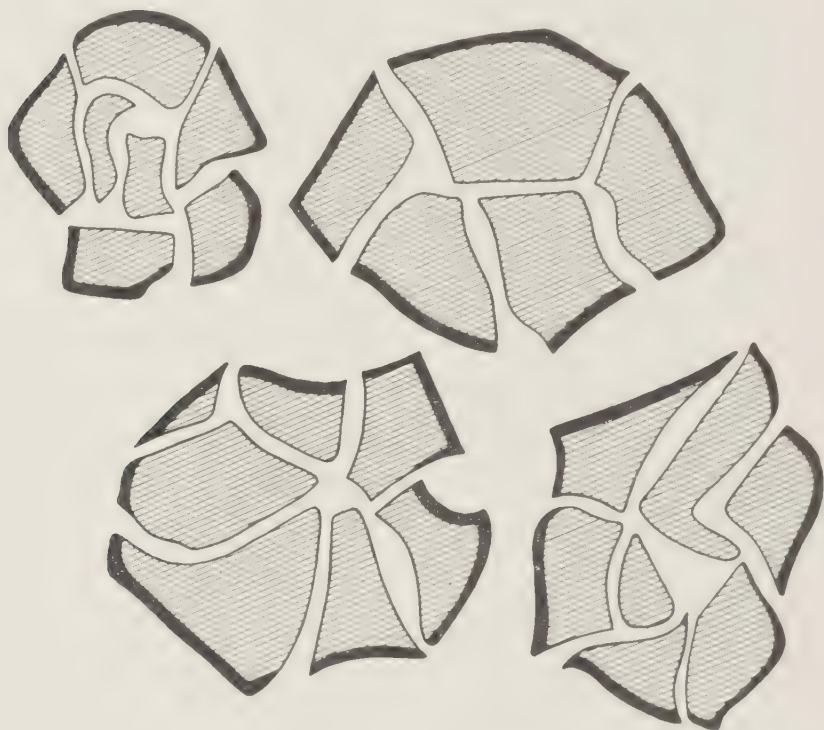
Figure 3. Soil crumbs

the most freely drained soil, and under very dry conditions, some water will be retained in the finer capillaries of the soil and some will be retained as an adsorbed layer all over the soil particles. It is in the water retained in the soil capillaries and in the water adsorbed on to the soil particles that the soil bacteria live. Aerobic bacteria will clearly flourish best under conditions of medium water content of the soil. If the soil is overfilled with water—i.e. is ' waterlogged '—the bulk of the pore space will be occupied by water that will preclude access

of air to the bacteria living in the interior of the soil particles. If the soil is too dry, the water film will be too thin and disjoined to allow 'lebensraum' adequate for the development
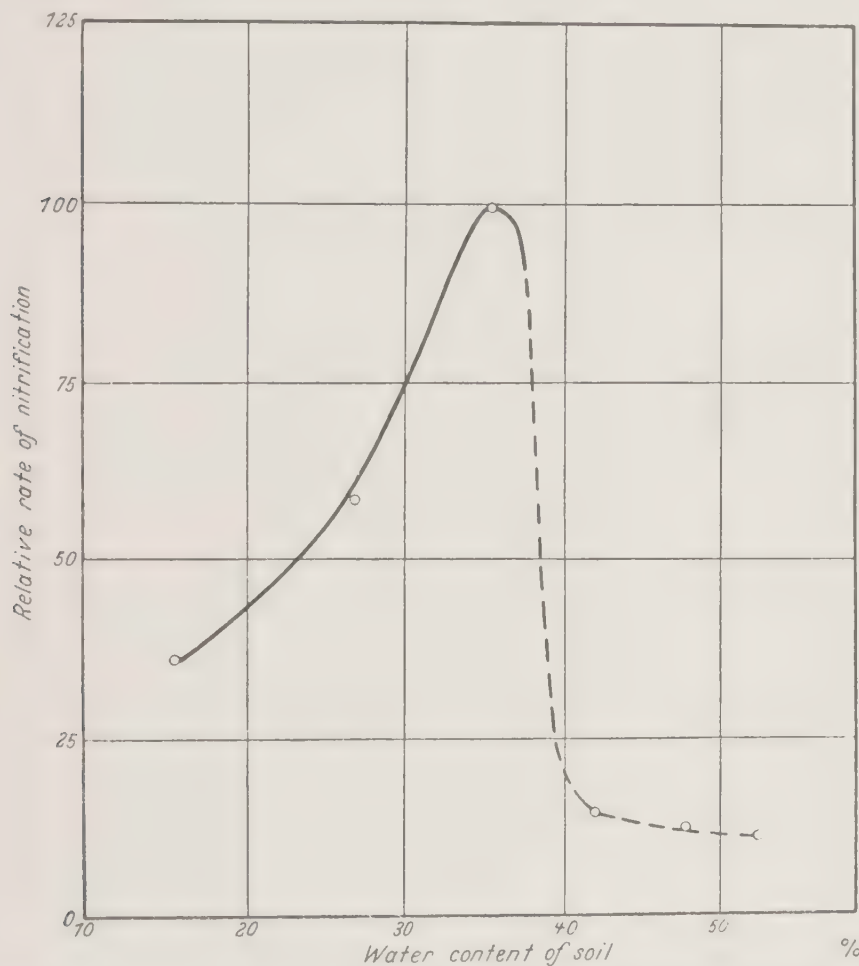
Figure 4. Soil water content and nitrification rate. Dotted line indicates visible water logging

of the organisms. This point is clearly brought out in *Figure 4*, which shows the relation between the observed rate of nitrification of added ammonium ions and the water content of a garden soil (data from author's own experiments). Nitrification, i.e. the conversion of ammonium ions to nitrate ions, is carried out in soil by the combined activities of *Nitrosomonas* and *Nitrobacter*,

two strictly aerobic, strictly autotrophic bacteria (*see* Chapter 5). *Figure 4* shows how the overall activity of the organisms increases as the water content of the soil is increased, that is, as they have more ' culture medium ' in which to develop ; at a certain point, however, the soil contains so much water that the air supply to the organisms is restricted and their activity drops very sharply to almost zero. These data were obtained in laboratory experiments on samples of the soil held in test-tubes, but they accord well with what is observed in the field. Nitrification is, *ceteris paribus*, most rapid in moist, drained soils of good crumb structure ; it is slow in very dry soils ; it does not take place in waterlogged soils.

The second property of soils that concerns us here is that of ' base exchange '. ' Base exchange ' is predominantly displayed by the clay fraction of the soil, although the soil humus may show it to some extent. This property is easily demonstrated. Take 100 c.c. of M/100 ammonium chloride, add 10 g of garden soil, shake, filter off the soil. Wash the soil in the filter funnel two or three times with water to free it of ammonium chloride solution. Combine the filtrate and washings and analyse them for ammonium ion. It will be found that only about half of the original ammonium ion is present, the rest has been retained by the soil. Now wash the soil in the funnel two or three times with, say, M/10 sodium chloride solution and combine and analyse these washings for ammonium ion. It will be found that the sodium chloride has displaced the ammonium ion from combination with the soil and that the washings contain the ammonium ion that was missing from the first analysis. These ' base exchange ' phenomena arise because the clay (and humus) particles behave as though they were large polyvalent anions ; they therefore attract cations to their surfaces, as shown diagrammatically in *Figure 5*. The upper half of this figure illustrates what happens when the ammonium chloride is first added to the soil ; the mixed collection of cations already present in the soil, and attracted to the surface of the clay or humus, is to some extent replaced by ammonium ions. In the lower half of the figure is illustrated the displacement of the adsorbed ammonium ions by the sodium ions of the sodium chloride washing solution.

This property of base exchange is important in soil nitrification because it has been shown, by means of the soil percolation technique, that the adsorbed ammonium ions, the ammonium ions held in the base exchange complexes of the soil, are attacked by the nitrifying organisms in preference to the ammonium ions free in the soil water (Lees & Quastel, 1946). In the soil percolation technique, there is a reservoir of metabolite solution (say ammonium chloride) and a column of soil ; small samples of the metabolite solution are removed from the reservoir, percolated through the soil, and returned to the reservoir (this is done automatically and there are devices to
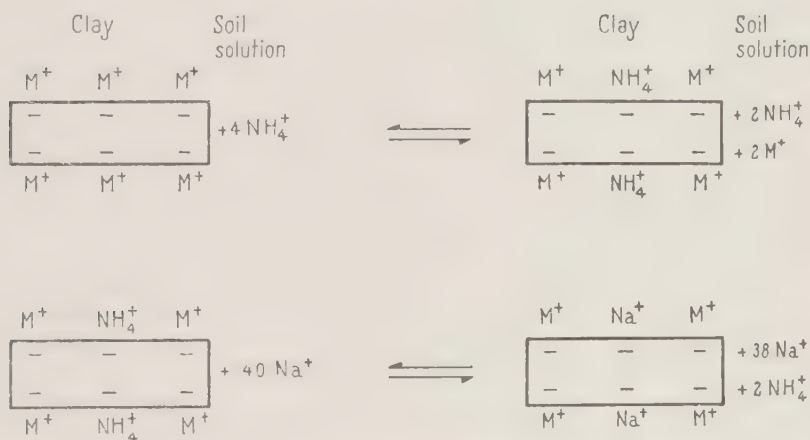
Figure 5. Base exchange

ensure that the soil does not become waterlogged). Chemical changes taking place in the soil are therefore reflected as chemical changes in the composition of the reservoir fluid (i.e. the percolating metabolite solution), always assuming that the changes are such as will affect the *soluble* constituents of the soil/solution system. If this assumption is valid, as it is in the case of nitrification of ammonium ions, periodical analyses of the percolating metabolite solution (usually referred to as ' the percolate ') can be used to follow the metabolism in the soil of the constituents of this solution. If one is following the metabolism of ammonium ions in soil by the percolation technique, it is possible to arrange for various initial distributions of the ions between the base exchange complex of the soil and

the percolate. One may begin with, say, 75 per cent of the ammonium ions adsorbed on to the base exchange complex of the soil and 25 per cent in the percolate ; or, with another sample of the same soil and with the same total amount of ammonium ion, one may begin with 25 per cent of the ion adsorbed and 75 per cent in the percolate. If one now follows the rate of nitrification of ammonium ions under these two sets of conditions, one finds that the rate is higher where the adsorbed ammonium ion is higher ; in fact, the rate is directly related to the proportion of ammonium ion adsorbed. This experimental finding is one basis of the statement that the adsorbed ammonium ions are nitrified in preference to ammonium ions in free solution.

The rates of nitrification that may be displayed by soils under percolation conditions are relatively enormous. An oxidation of $0 \cdot 2$ mg ammonium-nitrogen per day per gramme of soil is by no means unusual ; this corresponds, on the field scale, to the conversion to nitrate of one or two *tons* of pure ammonium sulphate per acre per day. It is therefore not surprising that in summer a fertile garden-soil is likely to have all its mineral nitrogen in the form of nitrate, even if it is dressed with ammonia-containing, or ammonia-generating, fertilizers. Nitrification, indeed, occurs very readily in most agricultural soils. In fact, it is true in general that the more readily a soil will nitrify added ammonium ions, the more fertile it is likely to be ; ' nitrifying capacity ' is a well established laboratory index of soil fertility. But this does not necessarily mean that a high nitrifying capacity is an agriculturally desirable characteristic of a soil ; all it means is that soil conditions that favour the growth of crops also favour the activities of the nitrifying organisms. These conditions are, for instance, adequate soil aeration, good crumb-structure, adequate water supply, more-or-less neutral pH reaction. But whether the activities of the nitrifiers themselves benefit plant growth is another matter. In the first place they lower the pH of the soil by converting a base (ammonia) into an acid (nitric acid) ; whether a lowering of pH is desirable will depend upon circumstances. At a lower pH, most trace elements will become more readily available to the plants growing in the

soil ; molybdenum will become less available. At a lower pH, the danger of scab infestation of potatoes will be reduced, the danger of club root of brassicas will be increased. In the second place, although the conversion of ammonia to nitrate means (for many plants) the conversion of mineral nitrogen from a less assimilable to a more assimilable form, it also means the conversion of the nitrogen from a form in which it is held, against the leaching action of rainwater, within the locus of the feeding roots (by the operation of base exchange phenomena) to a form (nitrate) in which it may easily be transported by rainfall downwards through the soil to the subsoil and so become lost to the plants. This loss of nitrogen from plant economy, a loss exacerbated by the activities of the nitrifying organisms, is a very serious one in regions of high rainfall and high temperatures such as are found in the tropics. There is little doubt that if we could discover sufficient about the detailed biochemistry of the nitrifiers to enable us (by suitable additions of the appropriate compounds to the soils) to quench their activities in tropical soils, tropical agriculture would benefit considerably.

At one time there was a deliberate attempt to utilize the action of the nitrifying organisms to further human ends. In the Napoleonic Wars the French, denied access to overseas saltpetre (sodium nitrate) deposits by the British Navy, resorted to the manufacture of saltpetre in specially prepared dung-heaps ; the manufacture of the saltpetre was an essential preliminary in the manufacture of gunpowder. The operation of these heaps depended first upon the conversion to ammonia of the various nitrogenous materials present (this was accomplished by the heterotrophic organisms present) ; but secondly it depended upon the power of the autotrophic nitrifying organisms to convert this ammonia to nitrate. How much gunpowder was ever manufactured as a consequence of this nitrifying activity is uncertain, but those interested in its manufacture may find a description of how to make a nitre heap in the First Edition of *Chambers's Encyclopaedia*.

Before leaving the subject of nitrification, there are two more questions. A soil may contain some 10^8 bacteria per gramme, mostly heterotrophic ; it has already been stated in Chapter 2

that some heterotrophs can form nitrite and that some can form nitrate. Are the activities of all these heterotrophic organisms capable of forming nitrite and nitrate totally unimportant in ' natural ' nitrification ? Is all nitrification in soil, sewage, and dung due to the activities of the autotrophic nitrifying organisms ? As far as is known at present, the answer to both these questions is ' Yes '. This affirmative is based on three experimental observations : (1) The pH range within which autotrophic nitrifying organisms best operate is the range within which nitrification proceeds best under natural conditions. (2) All inhibitors of nitrification in pure cultures of the nitrifying organisms also prove to be inhibitors of nitrification under natural conditions. (3) When studied in pure culture, no organisms have yet been discovered that are capable of producing nitrite and nitrate with the speed or in the quantity achieved by *Nitrosomonas* and *Nitrobacter*, i.e. the autotrophic nitrifying bacteria. According to present evidence there is no reason to doubt that these two organisms, or closely related ones, are responsible for all, or virtually all, nitrification under natural conditions.

Another autotrophic activity of agricultural importance is the oxidation of sulphur to sulphate. This oxidation, which is accomplished by various thiobacilli, *Beggiatoa*, and *Thiothrix* (*see* Chapter 3), results in a very profound fall in pH as sulphuric acid is produced from neutral elementary sulphur: the addition of sulphur to soils is commonly practised when some reduction in the pH of the soil is required. The sulphur, usually ordinary flowers of sulphur, is more easily handled than sulphuric acid itself and is not so drastic in its effect on the soil micro-organic population ; yet owing to the metabolic activities of the autotrophic sulphur-oxidizing bacteria of the soil, the addition of sulphur to a soil is equivalent to the addition of sulphuric acid. The reduction of soil pH by sulphur addition is preferable to its reduction by addition of ammonium salts because sulphur addition does not result, as does addition of ammonium salts, in a violent disturbance in the nutrition of the plants growing in the soil. Sulphur additions have proved useful in increasing the fertility of very alkaline soils. and also in decreasing the incidence of scab in potato crops.

Heterotrophic, as well as autotrophic, bacteria are capable of oxidizing thiosulphate (*see* Chapter 3) ; therefore it cannot be said with certainty that thiosulphate oxidation in soil is necessarily autotrophic. Gleen (1949) has, however, made the interesting observation that in soil percolation experiments the same micro-organic population seemed to be involved in the oxidation of thiosulphate and the oxidation of thiocyanate. This suggests that some organisms similar to *Thiobacillus thiocyanoxidans* may have been involved in Gleen's experiments.

Under anaerobic conditions, such as obtain in waterlogged soils and may obtain in clay soils with little large-pore space, the activities of the autotrophic sulphate-reducing bacteria (*see* Chapter 6) may assume some importance. These bacteria, *Desulphovibrio* spp. for the main part, are facultative autotrophs and can reduce sulphate to sulphide either at the expense of hydrogen (when growing autotrophically) or at the expense of ordinary organic substrates (when growing heterotrophically). According to Butlin & Postgate (1954), the *growth* of the organisms is far greater under heterotrophic than under autotrophic conditions ; on the other hand, the *rate* of sulphate reduction to sulphide is far more rapid when hydrogen is the reducing agent than when the reducing agent is some organic substrate. Butlin & Postgate are inclined to the view that the reduction of sulphate to sulphide, which commonly occurs under anaerobic soil conditions, may sometimes be an autotrophic reduction (i.e. a reduction at the expense of hydrogen oxidation) carried out by *Desulphovibrio* spp. that have *grown* heterotrophically. It may be noted parenthetically that there is no difficulty in accounting for a supply of hydrogen ; a number of anaerobic organisms are capable of producing it. This reduction of sulphate to sulphide in soils has considerable economic repercussions because it is responsible for the corrosion of buried iron pipes, e.g. gas pipes. There are two theories about the mechanism of corrosion and both suppose that the surface of the iron initially behaves as a pattern of tiny voltaic cells which rapidly become polarized by the accumulation of hydrogen. In one theory depolarization is said to be accomplished because the hydrogen is consumed by the sulphate-reducing organisms ; in the other, depolarization

is said to be accomplished by the sulphide formed by the organisms. In either case, depolarization results in solution of metallic iron. A discussion of these theories is presented by Butlin & Postgate (1954).

Autotrophic bacterial activity in lakes and streams

In lakes and streams, that is in environments into which light can penetrate, the activities of the photosynthetic sulphur bacteria become of importance.

Conditions in the deeper layers of lakes, in stagnant ponds, or even sluggish streams, may become so anaerobic, that considerable quantities of sulphides may be produced by the sulphate-reducing bacteria. It sometimes happens, when sulphides have been produced in this way, that a population of photosynthetic sulphur bacteria establishes itself and oxidizes the sulphide first to sulphur and then to sulphate. Sulphur production by photosynthetic bacteria under these circumstances may be considerable. Butlin & Postgate (1954) report that one small lake in Cyrenaica yields 100 tons of elementary sulphur annually, all of it formed, apparently, by the action of photosynthetic bacteria. It may be noted here that under anaerobic conditions where sulphate reduction occurs, any concomitant re-oxidation of the sulphide formed must be carried out by the photosynthetic sulphur bacteria because the other autotrophic bacteria capable of oxidizing sulphides (i.e. the thiobacilli) are strict aerobes and cannot oxidize at all under anaerobic conditions ; *a fortiori* it may be noted that the photosynthetic sulphur bacteria are strict anaerobes and can operate only under conditions in which sulphate reduction and sulphide formation are possible. The bacteria usually responsible for the photosynthetic oxidation of sulphide to sulphur are the purple sulphur bacteria, which are more vigorous in growth than the green sulphur bacteria. If a large crop of purple sulphur bacteria develops in a lake or stream, the water may take on a marked reddish colour ; sporadic growth of such crops in the past may have given rise to the various legends that exist of ' rivers turning to blood '.

An interesting sideline to the various biochemical investigations on sulphur utilization by micro-organisms is the discovery

that the sulphur isotope of mass 32 (^{32}S) is preferentially used by them. Isotopic analyses of sulphate, sulphur, and sulphide in different geological deposits have shown that deposits of sulphur and sulphide in formations more recent than 800 million years have an augmented content of ^{32}S. This suggests that these deposits, but not earlier ones, were formed as the result of microbiological activity. Since these deposits are nearly twice as old as the earliest fossils, and since, as far as we know, only autotrophic bacteria are capable of producing elementary sulphur from any other sulphur source, the isotopic evidence is thus in harmony with the view that the autotrophic bacteria are indeed very primitive organisms.

Other activities of the autotrophic bacteria

Apart from their activities in soil and in lakes, the autotrophic bacteria impinge considerably on everyday life in other ways.

City air has a high probability of being contaminated with various reduced sulphur compounds such as hydrogen sulphide and sulphur dioxide. As a consequence, the water film on stonework in cities is likely to contain sulphur compounds (sulphides and sulphites) capable of attack by thiobacilli. In fact, thiobacilli, by oxidizing these compounds in the water films on stonework to sulphuric acid, contribute considerably to the rotting of the stonework in cities. Thiobacilli have an equally disastrous effect on concrete, and it is noteworthy that the work of Parker & Prisk quoted in Chapter 3 was inspired by the rotting of concrete structures brought about by the activities of various thiobacilli. Nitrifying organisms, which convert neutral ammonium salts to nitric acid and the acid of the anion in the original salt, are equally damaging to concrete and stone. The disintegration of fire hoses used by Civil Defence workers in the last war was shown to be due to the oxidation, by thiobacilli, of the sulphur contained in the vulcanized rubber structure of the hoses. The decay of iron pipes (particularly in America) used to convey sulphurous waters from coal seams is usually due to the production of sulphuric acid, by thiobacilli, from less acidic compounds originally present in the water. It will be remembered that

T. ferro-oxidans (*see* Chapter 3) was isolated from such sulphurous waters.

The study of autotrophic bacteria in their natural environments

It is usually true to say that the proper biochemical study of the activities of an organism in its natural environment is possible only when that environment can be transferred to the laboratory and maintained in some predetermined standard state. Moreover, it is also usually true to say that, even if the environment (with the organism operating in it) can be transferred to the laboratory, a biochemical study of the activities of the organism is possible only if the chemical changes taking place within the environment, as a result of the activities of the organism, can be followed without disturbing the conditions obtaining in the environment.

We are here concerned with the activities of autotrophic bacteria. If it is wished to study the biochemistry of (say) the photosynthetic bacteria inhabiting a pond, transfer of the natural environment of these bacteria to a laboratory presents no difficulty. It is merely necessary to take a sample of the pond water and preserve it in the laboratory under the same conditions of illumination and oxygen tension as operated in the pond when the sample was taken. Moreover, since the environment (pond water) is, on the macro-scale, quite homogeneous, samples may be taken from it without in the least disturbing any biological equilibria that may have been established.

Certain autotrophic bacteria are, however, typically soil organisms, and if it is desired to make a biochemical study of these organisms in their natural environment, it is first necessary to devise a method whereby the chemical changes wrought in the soil by their activities can be studied in the laboratory. It is necessary, in other words, to devise a method whereby soil can be studied as if it were a metabolizing tissue. Now a method widely used in the study of animal tissues is the 'perfusion technique', in which some tissue, e.g. kidney, is perfused with a solution of metabolites and the solution issuing from the tissue is analysed. Chemical changes brought about in the perfusing fluid as a result of perfusion through the tissue

can then be ascribed to the metabolic activities of the tissue perfused. A rather similar technique, the soil percolation technique, can in fact be used for the study of soil metabolism. Originally, this technique owed its inspiration to the normal tissue perfusion technique and was called the ' soil perfusion ' technique. This description is, indeed, still widely used, but semantically ' soil percolation ' is better.

A brief description of this technique has already been given (p. 87), but, since the technique has been so widely used for the study of soil metabolism, particularly for the study of soil nitrification (overwhelmingly an autotrophic process in garden and agricultural soils), a rather fuller explanation is not out of place here.

Since the technique was originally described (Lees & Quastel, 1944), a number of soil percolators have been devised by different workers. Different designs have different advantages and drawbacks ; possibly the simplest to make and operate, and by no means the least satisfactory, is the one shown in *Figure 6*.

Air, at some 10 cm Hg pressure, is forced into the bubbler tube (1) and, bubbling through the reservoir fluid (2), which is a solution of the compound whose metabolism in the soil is being studied, traverses the lift-tube (3), a sample of sieved soil (5) held between glass-wool plugs in a test-tube with a hole in the bottom, and escapes to the atmosphere through the exit tube (6). In traversing this path, the air carries bubbles of reservoir fluid with it ; these therefore rise up the lift tube, percolate through the soil, and eventually drip back into the main bulk of the reservoir fluid. During percolation there is always a head of reservoir fluid in tube (4) equivalent to the pressure drop between the surface of the reservoir fluid and the exit of the test-tube containing the soil sample. Should the soil sample become waterlogged or clogged, this pressure drop, of course, increases, the head of fluid in (4) rises, the level of the reservoir fluid therefore falls, and percolation automatically ceases because the height of the mouth of the lift-tube (3) above the level of the reservoir fluid becomes so great that bubbles of fluid are no longer projected into the lift-tube. The apparatus is therefore to a considerable extent self-regulating and only a

very coarse adjustment of the initial height of the lift-tube (3) above the reservoir fluid is necessary. This tube may be pushed up and down in the bung to accommodate different initial volumes of reservoir fluid if so desired.

Rubber sleeve

6

5

Glass wool

3

4

1

2
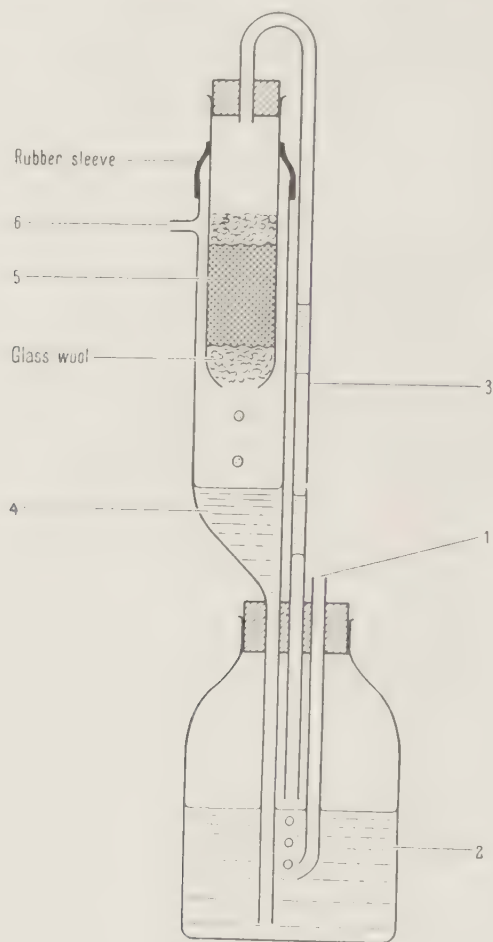
Figure 6, The Soil Percolator

Soil metabolism is followed by taking periodic samples of the reservoir fluid and performing on them the appropriate analyses. If, for instance, soil nitrification were being studied, a solution of some ammonium salt would be percolated and analyses performed from time to time for ammonium, nitrite, and nitrate ions.

The technique is a versatile one. It is possible, for instance, to percolate the soil sample with some metabolite and, when the metabolism is fully established, to remove the soil tube and replace it by an empty test-tube. If this is done when the metabolism of ammonium salts is under study, it will be found that, on removal of the soil, nitrification virtually ceases even though the reservoir fluid still contains plenty of ammonium ion. This is another demonstration of the fact, previously mentioned (p. 87), that soil nitrification takes place principally at the soil surface. Conversely, it is possible to percolate some compound A until its metabolism in the soil is fully established and then change the reservoir fluid to one containing a different compound B. If the organisms responsible for the metabolism of A are able to metabolize B in the same way as they metabolize A, metabolism of B will begin as soon as B is percolated; if not, there will be a lag before the metabolism of B begins. By this 'stimulated soil' or 'bacteria-saturated soil' technique, it has been shown that, in all probability, soil nitrification is an auto-trophic process. Soils in which nitrification has been well established, in which nitrification has been 'stimulated' by percolation with ammonium salts (and which are therefore 'saturated' with respect to their nitrifying population), nitrify a fresh percolate of ammonium salts immediately percolation is started; all other nitrogen-containing compounds either fail to yield any nitrification at all or else show a 'lag phase' during which a population capable of transforming them to ammonia is built up. (The behaviour of hydroxyl-amine, an intermediate in nitrification, is here anomalous, because it is chemically destroyed in soil and therefore yields no nitrite on percolation, even through a 'stimulated' soil.)

The soil percolation technique has been described at some length here because, although, as mentioned in the Introduction, it is not really well adapted to the study of the detailed bio-chemistry of autotrophic organisms, it can be used to study the general behaviour of autotrophic soil organisms in their natural environment. It has been simply adapted to measure the carbon dioxide production of soils (Lees, 1949 : this reference gives full details of how to carry out the technique) as well as

their oxygen uptake (Lees, 1950). The apparatus is easy and cheap to make and will function without any trouble, especially if a good garden soil is used and a little soil-conditioner is added to the percolate. Despite its drawbacks, it provides a very suitable introduction for any biochemistry student wishing to embark on a study of autotrophic bacteria.

Conclusion

This chapter is not meant to be exhaustive ; a comprehensive list of the literature on autotrophic activity in soil, sewage, natural waters, and other environments would probably occupy the whole of a book equal in size to this one. The purpose of this chapter has been to indicate to the reader, to whom the whole concept of autotrophic bacteria may be novel, that auto-trophic bacteria have a quantitative, as well as a qualitative (i.e. biochemical or philosophical), importance. They affect our agriculture, our chemical industry, and our buildings. They deserve our further attention.

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APPENDIX

THE CALVIN CYCLE

IT is now reasonably certain that the first step in green-plant assimilation of carbon dioxide is the addition of carbon dioxide to an 'active' phosphorylated 2-carbon fragment to give phosphoglyceric acid. If this 2-carbon fragment were derived from a (phosphorylated) pentose, two molecules of phosphoglyceric acid might be formed, one from the condensation of
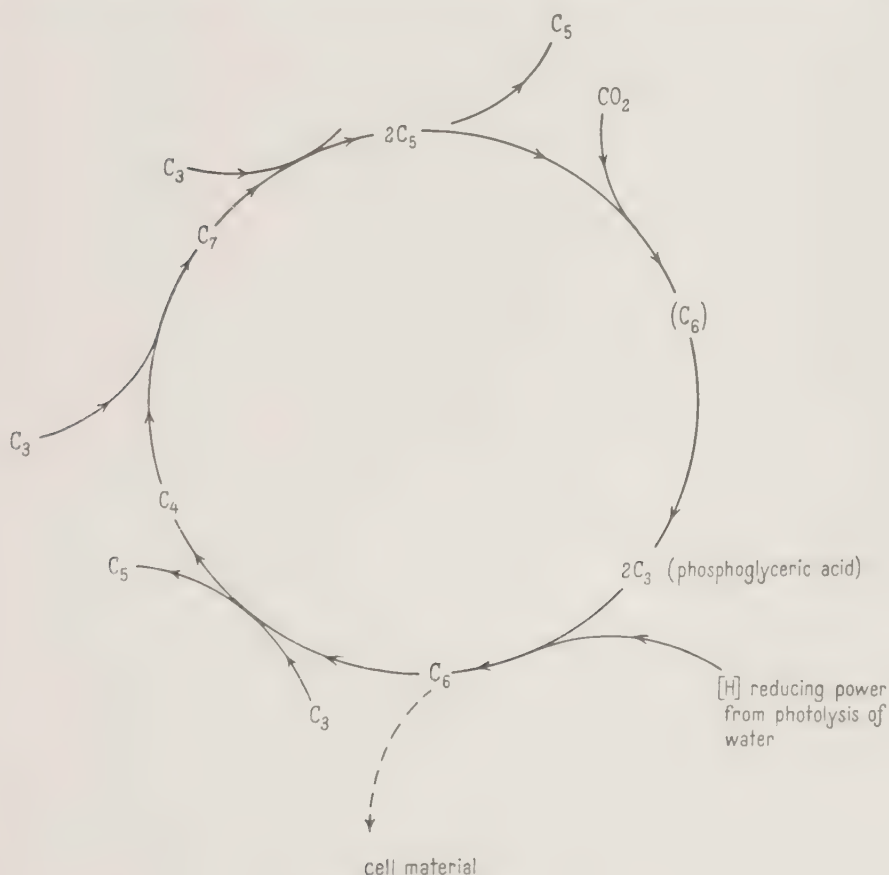
Figure 7. The Calvin Cycle

carbon dioxide with the 2-carbon fragment, one from the three carbon atoms remaining after removal of two carbon atoms from the pentose. The regeneration of the pentose is then envisaged as occurring by the interplay of various 3-, 4-, 5-, 6-, and 7-carbon carbohydrates mediated by such enzymes as transaldolase and transketolase. The sort of scheme that is thought to operate is outlined above (*Figure 7*) ; for simplicity, the phosphorylations and dephosphorylations involved have been omitted.

The experimental basis for the cycle is (*a*) the rapidity with which various compounds in the cell become ' labelled ' when the cells are allowed to perform a very short-term photosynthesis in the presence of carbon dioxide containing radioactive carbon, and (*b*) the observed distribution of the radioactive carbon in molecules such as hexoses formed as a result of such photosynthesis.

The scheme given here must not be thought of as accurate and proved in every detail ; it merely summarizes the *kind* of reactions involved. An interesting note on the enzymology of some of these reactions has been published by Racker (1955).

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INDEX

Thiosulphate *(cont.)*
— oxidation of, by sundry heterotrophic bacteria, 11, 23, 91
— — in soil, 91
Thiosulphite, role in *thiobacillus* metabolism, 23, 27
Thiothrix, in soil, 90
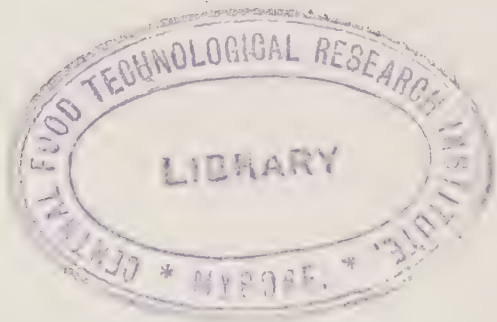— oxidation of sulphide by, 21, 90
TPN, in energy generating systems, 76 *et seq.*
— in nitrate reductase systems, 50
— transfer of hydrogen by, 7, 15

Vitamins *(see also* Growth factors), 5

Xanthomonas, capture of light energy by, 10

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...mistry